University of Alberta

A Newly Developed Migration Assay Shows Increased Monocyte Activity in Patients With Unstable Coronary Artery Disease

by

Michael Dean Kolodziejczyk ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

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To the scientists and scholars from days gone by, whose efforts I have endeavored to build upon, and to those pioneers of the future whose discoveries are yet to be realized.

ABSTRACT

Monocytes are critical to both the general inflammatory process and to atherosclerotic disease development. Macrophage invasion in unstable atherosclerotic plaque is known to initiate plaque rupture and thrombus (clot) formation. To study the relative activity of peripheral blood monocytes from patients with ischemic heart disease, a two chamber migration assay was modified, using the KG-1 and THP-1 cell lines and primary blood cell isolates. With this assay, monocytes from patients with severe unstable coronary artery disease, those patients with combined multi-vessel coronary artery disease and impending heart attack or unstable angina, were found to have increased monocyte migration in comparison with normal donors and patients with moderate disease. Lymphocyte migration remained low with all donors. The increase in the migration of peripheral blood monocytes may reflect increased cellular activation in peripheral blood monocytes *in vivo*. The results of this study provide additional support for monocyte activation in unstable coronary syndromes.

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LIST OF ABBREVIATIONS

ABBREVIATION	COMPLETE NAME
ABC	Avidin-Biotin Complex
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
CAD	Coronary Artery Disease
DAB	3-3'-Diaminobenzidine
DMEM	Dulbecco's Minimal Essential Media
ECG/EKG	Electrocardiogram
ECM	Extracellular Matrix
EDTA	Ethylenediamine Tetra-Acetic Acid
EST	Exercise Stress Test
ETT	Exercise Treadmill Test
FCS	Fetal Calf Serum
FMLPP	N-Formyl-Methionyl-Leucyl-Phenylalanine
γ-IFN	Gamma Interferon
HD	Heart Disease
IHD	Ischemic Heart Disease
IMDM	Iscove's Modified Dulbecco's Medium
IL-1	Interleukin-1
IL-2	Interleukin-2
LPS	Lipopolysaccharide
MØ(s)	Macrophage(s)
MHC	Major Histocompatibility Complex
MI	Myocardial Infarction
MRI	Magnetic Resonance Imaging

PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline Solution
PDGF	Platelet Derived Growth Factor
PLSD	Protected Least Significant Difference
RA	Rheumatoid Arthritis
SERPIN	Serine Proteinase Inhibitor
SMC(s)	Smooth Muscle Cell(s)
TGF-ß	Transforming Growth Factor-ß
TNF	Tumor Necrosis Factor

CHAPTER I - INTRODUCTION[1]

There is a growing consensus that inflammation is critically important in the development of atherosclerosis (Ross 1993). This is not a new idea; rather, it is an older idea to which we are returning. An area under active study is the importance of inflammatory cells in atherosclerotic plaque development, especially monocytes, endothelial cells and smooth muscle cells (SMCs). The monocyte, which is the precursor of the macrophage (mØ) (Smith 1990), is the class of cell which provides the greatest source of mature mØs at inflammatory foci (Gerrity 1981, Bylock and Gerrity 1988, Gerrity and Schwartz 1977). During an inflammatory response, monocytes are released from the bone marrow, circulate through the bloodstream until they encounter a site of tissue damage, migrate into the damaged tissue, mature into mØs, and undertake inflammatory functions (van Furth *et al* 1972, Nielsen 1990). Monocyte-derived mØs are the primary source of the foam cells that are highly characteristic of plaque development, and comprise a large component of the unstable shoulders of plaque (Ross 1995, Hansson *et al* 1989). They also play an important role in hyperlipidemia and through their invasion, and release of factors, are critical to plaque growth, breakdown, and rupture (Faggiotto *et al* 1984, Davies 1996).

It is because of the importance of monocytes and mØs in inflammation and because plaque development appears to be the result of a hyper-reactive inflammatory response (Ross 1986), that it makes sense to test for an increased level of activity in peripheral blood monocytes from patients with various degrees of atherosclerotic plaque development. Testing whether the activity of peripheral blood monocytes is increased in patients with unstable plaque development, is the primary objective of this thesis (see Section I.VIa).

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[1] A version of this chapter has been submitted for publication.

Perhaps, the best way to gain an understanding of the general pathological processes of atherosclerosis is to view today's beliefs in light of their historical context. Therefore, before I specifically focus on the importance of the monocyte, a critically important inflammatory cell in atherogenesis, I will highlight the historical views that have coloured our present understanding of atherosclerotic disease.

I.I. HISTORICAL

The term atherosclerosis was originally coined by F. Marchand (1904) in order to describe a form of artery disease that combined fatty changes with sclerosis. The root of the word atherosclerosis is derived from the Greek words *athero*, meaning gruel or paste, and *sklerosis*, meaning hardness. The pathogenesis of this condition involves the deposition of fatty substances, cholesterol, cellular debris, connective tissue (*ie* collagen and elastin), calcium and fibrin in the inner lining of an artery. The tissue proliferation that results in plaque growth. Atherosclerotic plaque narrows the lumen of the affected artery. In turn, blood flow is potentially decreased to a point where myocardial infarction (heart attack), cerebrovascular accident (stroke), and/or peripheral vascular disease (such as gangrene) occurs.

Atherosclerosis, the most common form of artery disease, is recognized as one of the leading causes of morbidity and mortality in industrialized countries. In these countries, ischemic heart disease is the principle cause of death among all of the circulatory diseases which, in turn, cause approximately one-half of all deaths (Lopez 1990). Atherosclerosis is not new however. It has afflicted humanity for eons, as is demonstrated in cases dating back to the Egyptian mummies (Ruffer 1911).

Atherosclerosis was described over a century ago, by Rudolf Virchow, as a chronic irritation of the vessel wall (Virchow 1856); a view that many scientists are returning to today. Virchow believed that inflammation was

the primary factor in atherogenesis which then promoted the overgrowth of the intimal connective tissue. This was followed secondarily by a fatty generation (referred to today as intimal hyperplasia). He believed that this process initiated with an irritation of the intima caused by the pressure of circulating blood along critical points of the artery, a hypothesis which is supported by recent studies (Glagov *et al* 1988, Yamamoto *et al* 1996, Friedman *et al* 1993, Nguyen and Haque 1990). Progressive plaque thickening eventually interfered with the nutrition of intimal cells, which naturally led to fatty generation. History has shown that Virchow's hypothesis, some 140 plus years old, was correct.

Aschoff (1907) later discovered that the fatty material in the diseased artery (plaque) was largely composed of cholesterol esters. Following this discovery, Aschoff (1914) came up with the first modification of Virchow's theory. His description of atherogenesis essentially stated that functional wear and tear, or excessive stresses, caused a loosening of the "ground substances" (*ie* basement membrane) of arteries together with a proliferation of "other factors". Ground substance swelling initiated in the deep tissue of the intima and was accompanied by molecular disintegration. Plaque, therefore, resulted from a functional deterioration of the artery (Aschoff 1914). Chemical changes and necrosis then led to the formation of calcified plates and ulcerations which represented the end links of a continuous chain.

While Aschoff's model was more encompassing than Virchow's, it was not as accurate based on what we know today. Unfortunately, many investigators adopted Aschoff's theories in the 1940's (Leary 1941) which slowed progression of the understanding of atherosclerotic pathology.

Klotz (1914, 1915) attempted to bring the scientific school of thought more into line with what is understood today with his insistence that fat was not deposited by a mechanical process. Rather, he felt that mistakes were being made by studying advanced lesions, instead of the smaller fatty and fibrous early lesions. This lead to his once famous dictum "A study of the

earliest stages of the lesions in the tissue of man is most secure upon which to base conclusions." (This makes equal sense today, since we must first understand the etiology of atherosclerosis in order for us to fully comprehend the disease process.) His approach demanded a study of the earliest stages of the disease. Klotz accomplished this through the study of the less developed lesion in early atherosclerotic plaque.

Klotz's (1914) studies used arterial sections that were obtained from individuals who succumbed to bacterial infections. Klotz felt that atherosclerotic plaque growth was induced by microbial toxins, the bacterial infections directly, or some altered physiological state that occurred during the fighting off of an infection. He used specimens from patients with scarlet fever (streptococcus), pneumonia (pneumococcus), dysentery (Bacillus), and other infectious organisms. Several other causes that were believed to be atherogenic in his day included tobacco, lead, adrenaline (epinephrine), and mechanical stress derived from high blood pressure (Klotz 1914, 1915). Several of these factors are still considered to be risk factors today (see page 9).

Leary (1934) did some very interesting work on the pathology of atherosclerosis which encompassed the very early to the severely advanced stages of the disease. His approach was to study samples from subjects ranging in age from 6 months (*ie* early plaque development) to the advanced plaques in the very old (Leary 1934, 1936). He showed that atherosclerosis of the coronary arteries in youth was marked by an increase in subendothelial connective tissue. This connective tissue tended to be of loose texture with little or no collagen formation. In older subjects (>40 years of age) collagen became evident and true scar tissue appeared (*ie* progressive plaque formation). Advanced lesions (from the elderly) exhibited persistent scar tissue, which was often present in the intima. Lipid-laden foam cells (mØs) accumulated 'en masse' with little fibrous support and, therefore, little provision for the nutrition of cells. This lead to widespread necrosis in the advanced lesions and the production of atheromatous abscesses.

In youth, the earliest atherosclerotic lesions showed lipid material which manifested itself as fine droplets in the "ground substance" of the endothelial cell layer. Leary (1936) showed an interesting correlation between the progression of this lesion, and the presence of so called "globular lipophage" (hereunto referred to as monocyte derived foam cells). Not only was it the first cell to appear but, if a lesion resolved, the numbers of cells declined from numbers that could initially form a continuous layer along an active lesion, to numbers that could only form focal collections in the surface layer, to a final elimination of these cells at lesion resolution. MØ egress from lesions has been demonstrated more recently in electron microscope studies (Davies 1988, Faggiotto and Ross 1984, Gerrity and Naito 1980). According to Leary (1936), in a resolving lesion, monocyte derived foam cells tended to "necrobiose" which was marked by cytoplasmic disintegration, fusion of lipid into larger irregular drops, and degeneration of the nucleus.

Leary's description of the 'necrobiotic' process appears to be very similar, or identical, to what is termed apoptosis today (Ellis *et al* 1991). It is quite possible that the "hot" current topic of apoptosis was described over 60 years ago. It also appears that Leary's work, in the 1930's, correlates with current studies. Examples are seen in several of the recent immunohistochemical studies of apoptosis in atherosclerotic lesions (Isner *et al* 1995, Hegyi *et al* 1996, Libby *et al* 1996, Mitchinson *et al* 1996, Bjorkerud and Bjorkerud 1996).

The early histological studies, such as the ones that are covered above, are the basis for what we know today. However, they only serve as the foundation for later advances. It is this later work, particularly the work on the monocyte, on which I will now focus.

I.II. ATHEROSCLEROSIS: A MODERN VIEW

Atherosclerosis is an amazingly complex process in which the exact etiologic and pathogenic processes are still unknown today. However, several

theories have been proposed. Most researchers currently follow modifications of the Ross (1986) "Response to Injury" hypothesis which views atherogenesis as an inflammatory process that is initiated by endothelial or SMC damage. In fact, atherosclerotic vascular disease shares several traits with the inflammatory process. MØs and T cells are present in both (Emeson and Robertson 1988, Jonasson *et al* 1986, Libby and Hansson 1991, Gown *et al* 1986, Munro *et al* 1987, Drew and Tipping 1995), as is MHC Class II expression (Hansson *et al* 1986, Libby and Hansson 1991). Other factors which are correlated include: fibrosis, humoral elements such as complement, and increased vascular permeability (reviewed by Seifert and Kazatchkine 1988).

Atherogenic cellular damage differs from the damage that arises during an acute arterial injury, which would normally undergo lesion resolution. The primary difference in these lesions, is that the source of the atherogenic arterial injury is not a single insult. Rather, it is a chronic, or at best episodic injury, which results in the progression of a fatty streak into a fibrous plaque (reviewed by Ross 1993). Removal of the insult can lead to the reversal of atherosclerotic lesions. Faggiotto and Ross (1984) documented this in an electron microscopic study on primates where a one month hypercholesterolemic diet was followed by nine months of normal diet. They found that a fifty percent decrease in the number of arterial segments that contained subendothelial lesions was present in primates with restored low cholesterol diets upon comparison with hypercholesterolemic controls. Several clinical trials have also shown that a reduction in hypercholesterolemia in human heart patients can improve atherosclerotic lesion pathology (Maher 1995, Simons 1994, 4S Group 1994, Shepherd *et al* 1995). Waters and Lesperance (1991) clearly stated "...interventions lowering LDL cholesterol or raising HDL cholesterol retard the progression of coronary atherosclerosis, promote regression and thus decrease the incidence of coronary events."

One step in atherogenesis is the production of the fatty streak which consists of lipid-rich (cholesterol-laden) mØs and T-cells in the intima of a

blood vessel (Munro *et al* 1987, Libby and Hansson 1991). Faggiotto *et al* (1984) illustrated the developmental sequence of atherogenesis using electron-microscopy time-course experiments in primates. In this hypercholesterolemic study, one of the most comprehensive electron microscopic time-course studies to date, several stages in plaque development were shown:

1) At the earliest time points in their experiments, numerous areas were found in the aorta where leukocytes, predominantly monocytes, adhered to the endothelial cell layer. These monocytes appeared to migrate along the surface of the endothelium and also appeared to diapedese through the endothelial cell layer into the intima (Faggiotto *et al* 1984). This is complemented by other studies (Joris *et al* 1983, Jerome and Lewis 1984) where circulating monocytes were attracted to a site of arterial injury and attached to the vascular endothelium (a process called margination).

2) Focal protrusions and subendothelial lipid-laden mØs began to appear, in arterial segments, as an undefined insudate which was covered by intact endothelium. (According to Faggiotto and Ross (1984) clusters of subendothelial mØs represent the earliest form of the fatty streak.) The mØs that appeared in early lesions were more numerous, contained numerous lipid deposits, and were enlarged two to three fold compared to normal cells. Lesions predominantly appeared in the aortic arch, aortic branches, and aortic bifurcations (Faggiotto *et al* 1984). Similar distributions of lesions were found by other authors (Yamamoto *et al* 1996, Friedman *et al* 1993).

3) Fatty streaks macroscopically visible over time due to the accumulation of foam cells. The early fatty streak lesions often contained multi-layered foam cells, some of which were 4 to 6 times larger than normal mØ (control) cells. Below the foam cells, lipid-laden SMCs appeared and, as the number and diameter of lipid-laden mØs continued to increase, layers of mØ derived foam cells were produced. The lesions were generally still covered by an intact, though highly irregular, endothelium (Faggiotto *et al* 1984).

4) As fatty streak formation progressed, endothelial dysfunction became apparent. Advanced fatty streaks demonstrated an irregular morphology with an endothelial layer that was stretched extraordinarily thin. Areas of focal separation appeared which exposed the underlying arterial connective tissue and cells to the lumen of the artery, and the circulating blood, which provided a means for potential thrombus formation (Faggiotto *et al* 1984).

Endothelial separation leads to the loss of some foam cells and allows for platelet adhesion to the remaining exposed foam cells. Interestingly, Faggiotto *et al* (1984) reported that some foam cells appear to exit fatty streaks by diapedesis through an intact endothelium. This complements earlier work (Poole and Florey 1958, Gerrity 1981, Gerrity 1981a, Gerrity and Naito 1980) which suggested that an egress of mØs from plaque could occur. When enough foam cells exit a lesion, the connective tissue is exposed. This leads to the appearance of multiple layers of adherent platelets over regions of exposed connective tissue and cells (Faggiotto and Ross 1984).

In further studies, with more advanced fatty streak lesions, strands of collagen and elastin were found around the SMCs that normally are present beneath the macrophagic foam cell layer (Faggiotto and Ross 1984). Several animal studies have shown that advanced fatty streak lesions precede the development of intermediate lesions (Faggiotto and Ross 1984, Faggiotto *et al* 1984, Dartsch *et al* 1989, Hansson *et al* 1991, Libby and Hansson 1991).

Intermediate lesions, which represent an extension of fatty lesion development, are composed of layers of mØs, SMCs and, inside the luminal third of the artery, several types of inflammatory cells which are primarily lymphocytic in nature. However, some neutrophils, basophils and mast cells have also been reported (Faggiotto and Ross 1984, Kovanen *et al* 1995). Fibrous connective tissue , SMCs, and inflammatory cells become common with time. Further lesion progression leads to fibrous plaque development which is composed of monocyte derived mØ cells, SMCs, and T cells (Jonasson *et al* 1986, Libby and Hansson 1991) as well as areas of necrosis.

Advanced plaques become vascularized and necrotic areas appear which contain cholesterol, lipid, calcium deposits, a few SMCs, and cellular debris (Hansson *et al* 1989, Faggiotto and Ross 1984, Stary 1989, Ross 1995). Most of the extracellular cholesterol in these plaques appears to come from the rupture of mØ (foam) cells, apoptosis of cells which border the necrotic region (Geng and Libby 1995), and from the accumulated debris from cell death (Goldstein and Brown 1977). A necrotic core can also form from a coalescence of smaller lipid particles (Stary 1989). Interestingly, foam cells tend not to lie inside the necrotic core which is generally acellular. Instead, they border the lipid core (Stary 1989, Ball *et al* 1995, Davies 1996).

For atherogenesis to be initiated, an injury must first occur (Ross 1986). One can view each of the known risk factors for the development of coronary artery disease as a possible source of endothelial injury. These risk factors include: elevated levels of cholesterol and triglyceride in the blood (National Institute of Health 1971, Keys *et al* 1984, Law *et al* 1994, Waters 1994), hypertension, also known as high blood pressure, (Nerem and Cornhill 1980, Karino *et al* 1987, Glagov *et al* 1988, Stamler *et al* 1985, Thelle 1992), Diabetes Mellitus (Garcia *et al* 1974, Bierman 1992), Homocyst(e)inemia (Bowen *et al* 1987), autoimmune reactions (Cerilli *et al* 1985, Hansson *et al* 1989), viral or bacterial infections (Klotz 1914, Benditt *et al* 1983, Fabricant *et al* 1983, Visser and Vercellotti 1993, McDonald *et al* 1989), and cigarette smoke (Becker *et al* 1976, Thelle 1992, Tverdal 1989). In Canada, Australia, and the United States of America, where health promotion campaigns to the lower cardiovascular risk factors for have been used for many years, the death rate from ischemic heart disease has dropped 30-40% over 25 years (Lopez 1990).

Interestingly, plaque is more prone to develop in some arteries while it is far less likely to develop in others. Further, atherosclerosis is more likely to develop in predictable areas of that artery. The carotid bifurcations, coronary arteries, and vessels supplying the lower extremities are at the highest risk of atherogenesis and, within these arteries, areas of bifurcations, branches, and

curvatures are at increased risk (Glagov *et al* 1988, Faggiotto *et al* 1984, Yamamoto *et al* 1996, Cornhill *et al* 1985). This has lead to the hypothesis that mechanical wall stress, shear stress, and mural tensile stress can all potentiate atherosclerotic development (Glagov *et al* 1988).

One explanation for the tendency of atherosclerotic lesions to be found at bifurcations and regions of curving artery is the local hæmodynamic environment which varies from site to site in the blood vessel (Yamamoto *et al* 1996). This environment depends on a limited number of variables, especially flow rate with time and vessel geometry (Friedman *et al* 1993). Unlike the systemic risk factors listed above, geometric risk factors act locally. Friedman *et al* (1993) showed that the branch angle between the left circumflex and left anterior descending arteries may be correlated with the severity of artery disease due to differences in hæmodynamic stress. Similar results were found by Nguyen and Haque (1990). In the renal artery, atherosclerotic plaque develops primarily in the section of artery that lies 1-2 cm downstream of the renal ostium. Shear stress, from turbulent blood flow at this site, appears to be correlated with atherogenesis in this region (Yamamoto *et al* 1996).

After initial endothelial cell damage by one of the above risk factors, fats, cholesterol, fibrin, platelets, cellular debris and calcium are deposited in the artery wall over time. This damage may stimulate cells in the arterial wall to produce cytokines which results in the accumulation and proliferation of additional cells, such as mØs and SMCs in the growing plaque (Ross 1995). These migrating cells may accumulate and divide while fat builds up within and around them. Connective tissue then forms and the innermost layer of the artery (the intima) markedly thickens into plaque.

As atherosclerotic lesions grow to be more complex and occlusive, they often will obstruct the flow of blood (Fuster *et al* 1992). If the plaque growth is sufficient, the diameter of the artery will be reduced to a point that blood flow becomes insufficient and results in ischemia (decreased oxygen supply to the tissues). When blood flow through a coronary artery is completely stopped by

thrombus (blood clot) formation, a heart attack (myocardial infarction) occurs. Similarly, if the oxygen supply to the brain is cut off, a stroke occurs, while loss of blood flow to the extremities causes ischemia in the limbs and ultimately results in gangrene.

The dense lesions of fibrous plaques become covered by a cap of dense collagen connective tissue. This connective tissue surrounds embedded SMCs, which are rich in rough endoplasmic reticulum content (Stary 1989). A predominantly fibrous muscular cap helps characterize stable plaque. This fibrous cap, which arose from the prior proteoglycan layer, overlays a relatively small core of lipid and necrotic debris (Stary 1989, Ross 1995).

Unstable plaques generally differ by possessing a substantial lipid core, increased numbers of mØs (Fuster *et al* 1992), and mØ rich shoulders (Jonasson *et al* 1986). Unstable plaques may rupture leading to a heart attack, stroke, or other complications. In these unstable plaques, which often are composed of a soft and deformable lipid core, hæmodynamic stress must be disproportionally carried by the cap. When this cap is thin and/or uneven, the hæmodynamic stress is greatly increased contributing significantly to the likelihood of plaque rupture (Davies 1996). When this rupture occurs, it is associated with an inflammatory process (van der Wal *et al* 1994). It is not known whether activation of monocytes at sites of unstable atheroma is associated with a generalized activation of circulating blood monocytes.

I.III. IMPORTANT CELL POPULATIONS IN ATHEROGENESIS

I.IIIa. Endothelial Cells

Endothelial cells normally provide a single cell thick lining along the inside of an artery. They prevent monocytes, other leukocytes, and platelets from arbitrarily binding to the connective tissue of an artery and, in doing so, prevent thrombus formation and tissue damage (Ross 1995). Through the

up-regulation of various receptors, the endothelium is also involved in the attraction and adhesion of leukocytes to areas of active inflammation.

The injury of endothelial cells, or possibly SMCs, is essential in the initiation of atherosclerosis. This theory was formally advanced for the first time by Ross and Glomset (1973). In fact, the injury of endothelial cells by such factors as hæmodynamic stress, and the resulting cellular dysfunction, can cause increased arterial lipoprotein uptake (Mora *et al* 1989) and alterations in gene expression (Nagel *et al* 1994). Endothelial injury also up-regulates several different cellular adhesion markers to which monocytes and T cells bind in early fatty streak formation (Ross 1995). This results in an influx of leukocytes from the circulating blood into the arterial wall.

One of the first changes during atherogenesis is the up-regulation of different adhesive glycoproteins on the endothelial cell surface. ICAM-1 staining is dramatically increased in arteries during plaque formation. Poston *et al* (1992) showed that endothelial ICAM-1 expression was weak in normal non-stimulated arteries and is markedly increased during atherogenesis. Very intense ICAM-1 expression also occurred in the mØs and SMCs which were contained in plaque. The pattern of expression paralleled that of inflammation (Pober *et al* 1992).

The ICAM-1 receptor is the CD11/CD18 heterodimer which is present on virtually all circulating white blood cells (Kishimoto *et al* 1989, Marlin and Springer 1987). The CD18 ß-subunit is non-covalently linked to one of three α-subunits which could be CD11a (LFA-1), CD11b (Mac-1, Mo1, CR3), or CD11c (p150). All of these heterodimers are present on monocytes. Expression of CD11b and CD11c is increased upon cellular activation. However, this change does not necessarily correlate with increased phagocyte adherence to the endothelium (reviewed by Carlos and Harlan 1990).

VCAM-1 expression is also increased during plaque formation on mØ, SMCs, and on endothelial cells (O'Brian *et al* 1993). A homologue of VCAM-1 is up-regulated in the hypercholesterolemic rabbit, even prior to monocyte

and T lymphocyte influx into intima of the artery (Cybulsky and Gimbrone 1991). The receptor for this is the (CD49dCD29) very late antigen-4 (VLA-4) β_1-integrin.

Areas can exist in intermediate plaques where the endothelial cells are retracted. This exposes the underlying lipid-laden mØ and monocyte derived foam cells which, in turn, allows for platelet binding and thrombus formation (Davies *et al* 1988, Davies 1996, Faggiotto and Ross 1984, Faggiotto *et al* 1984). Prior to the retraction of these cells, the endothelial cell layer can be stretched extremely thin. Injury to the endothelial cell layer may arise mechanically from the stretching of endothelial cells, or from the release of factors from the underlying mØs (Nathan *et al* 1979, Nathan *et al* 1980, Faggiotto and Ross 1984, Faggiotto *et al* 1984).

Simionescu *et al* (1986) demonstrated that cholesterol uptake into the arterial intima can be performed by endothelial cells prior to the appearance of morphological changes in the artery. This may even precede the appearance of monocytic cells. Oxidation or glycosylation of cholesterol, by the endothelial cells, may occur during endothelial transit (transcytosis) which, in turn, can initiate the atherogenic process (Steinberg 1991). Generation of reactive oxygen molecules by endothelial cells (Matsubara and Ziff 1986) can also lead to modification of the subendothelial connective tissue matrix (Greenwald and May 1979) and to monocyte chemotaxis (Quinn *et al* 1987).

I.IIIb. Monocytes

The monocyte is the precursor of the mØ. After exiting the bone marrow, monocytes circulate through the blood until they infiltrate the tissue (Smith 1990). Once in the tissue, monocytes rapidly differentiate into mØs and initiate their regulatory functions. In the artery, these functions can include scavenging for bacteria or cholesterol which was deposited into the intima of the artery by endothelial cells. After phagocytosing endothelial

processed lipoproteins, mØs release toxic substances which can potentiate arterial damage (Henriksen *et al* 1981, Henriksen *et al* 1983).

Monocytes are present during atherogenesis from earliest fatty streaks through to the most advanced fibrofatty lesions (Klurfeld 1985, Ross 1995, Davies 1996, Hansson *et al* 1991). In fact, the first morphological change in atherogenesis is the adhesion of monocytes (and T cells) to the endothelial surface (Watanabe *et al* 1995, Faggiotto *et al* 1984), followed by migration into the intima (Joris *et al* 1983, Davies *et al* 1988, Faggiotto *et al* 1994). In several animal models, mØs have been detected beneath the endothelial layer of the artery prior to the formation of atherosclerotic lesions (Faggiotto *et al* 1984, Lee *et al* 1970, Joris *et al* 1979, Massmann and Jellinek 1980). This suggests that monocytes normally have a scavenger function in arteries, acting to remove cholesterol, or other possible forms of insult, as occurs in other tissues (Faggiotto and Ross 1984, Gerrity 1981, Massmann and Jellinek 1980). This role may be amplified in hypercholesterolemia (Faggiotto *et al* 1984).

Although some fatty streaks may be initiated through pre-existing intimal mØs, most mØs in atherosclerosis are derived from circulating peripheral blood monocytes (Faggiotto *et al* 1984, Gerrity 1981, Gerrity 1981a, Gerrity *et al* 1979, Stary and Malinow 1982). Peripheral blood monocytes preferentially bind to injured endothelium (Hansson *et al* 1989). Post-endothelial injury, monocytes, and some T cells are the first cells that penetrate through an injured endothelium during the initial stages of atherogenesis (Faggiotto *et al* 1984, Faggiotto and Ross 1984, Stary 1989). Once through the endothelial cell layer, monocytes differentiate into mØs, acquire fats and cholesterol, further differentiate into foam cells, and eventually dominate the early developing human fatty streak (Aqel *et al* 1985, Munro *et al* 1987, Ross 1993). At least some of these fatty streak lesions progress into fibrous plaques (Faggiotto and Ross 1984, Faggiotto *et al* 1984).

Electron microscopic studies have shown that in intermediate plaques, some lipid-laden mØs appear to exit from the progressing plaque by passing

through endothelial cell junctions, as mØs are known to do in other inflammatory conditions (Faggiotto and Ross 1984, Faggiotto *et al* 1984, Davies *et al* 1988, Gerrity 1981a, Gerrity and Naito 1980). This results in increased numbers of foam cells in the blood during lesion progression. These foam cells, which lose most of their ability to adhere to plastic, increase in primates from 2 per 1000 white blood cells to an average of 48 per 1000 white blood cells within a year of starting an atherogenic diet (Faggiotto and Ross 1984).

Porcine studies have demonstrated that monocytes can enter very early atherosclerotic lesions and pre-atherosclerotic areas of pigs by penetrating the endothelium and taking up cholesterol in the intima (Gerrity 1981). Peripheral blood monocytes continue to attach to many of the fatty lesions while lipid-laden mØs appear to exit the lesion. This suggests the presence of a dynamic process where some mØ derived foam cells exit the growing atherosclerotic lesion as new monocytes enter (Faggiotto *et al* 1984, Faggiotto and Ross 1984, Gerrity and Naito 1980, Gerrity 1981, Gerrity 1981a). It therefore appears that the removal of lipid and cholesterol by monocytes could be part of a normal healing process (Gerrity 1981, Faggiotto and Ross 1984).

Gerrity (1981) was the first to hypothesize that monocytes normally provide an important defense mechanism, against cholesterol-induced atherosclerosis, via the clearance of cholesterol from the arterial wall by monocytes and mØs. Gray and Shankar (1995) later discovered that CD11b and CD18 expression is decreased in atherosclerotic lesion derived mØs. They suggested that this potentially could prevent the egress of mØs from plaque. In doing so, the clearance of cholesterol from plaque would be inhibited, thereby affecting the development of plaque. While their work was preliminary, it complements our limited understanding of this process.

Monocyte derived mØs may be particularly important in the unstable regions of plaque. This shoulders of plaque are highly prone to rupture which can lead to hemorrhage, thrombosis, and occlusion of an artery (Davies and Thomas 1984). When one considers that monocytes and mØs predominate

in the unstable shoulders of fibrous plaque (Jonasson *et al* 1986), their relative importance in unstable coronary syndromes is emphasized.

Monocyte derived mØs may also appear in the fibrous cap of many types of unstable plaques which, in turn, disrupts the densely woven collagen and SMC matrix cap that is indicative of more stable plaques. In doing so, mØs weaken the overall strength, and therefore stability, of advanced plaque (Lendon *et al* 1991). When mØ numbers are increased, while the expression of tissue factor is increased, and SMC numbers are decreased, a thin or uneven fibrous cap structure is present, and a proportionally large lipid core is present in a plaque, the instability of plaque is greatly increased (Davies 1996, Fuster *et* al 1992, Davies and Thomas 1984). This substantially increases the chance of plaque rupture and thrombosis. MØs can make a bad situation worse through the release of proteases that are capable of degrading the connective tissue matrix which normally acts to stabilize the structure of plaque (Dollery *et al* 1995). Active proteolytic activity has been detected in freshly isolated plaques which may trigger the "self-destruction" of the atherosclerotic lesion (Davies 1996).

I.IIIc. Lymphocytes

Historically, little attention has been paid to the lymphocyte in the development of atherosclerosis. In fact, until very recently, immunological phenomena have been viewed as being secondary in importance to other atherogenic processes (Wick *et al* 1995). It was only in 1986, that Jonasson *et al* first demonstrated that significant levels of T cells were present in plaque. In their study, T lymphocytes comprised ~20% of the fibrous cap, 9% of the lipid core, 22% of the shoulder, and 8% of the intima of fibrous plaque. In complicated carotid plaque, T cells were predominantly $CD4^+$ which contrasts with earlier stages of atherosclerotic development where $CD8^+$ T cells predominated

(Emeson and Robertson 1988, Munro *et al* 1987). The presence of B-cells in plaque is minimal (Jonasson *et al* 1986, Libby and Hansson 1991).

Because T cells have been found in association with monocytes/mØs and SMCs in plaque, it has been suggested by several authors that an immune process is involved in atherogenesis (Jang *et al* 1993, Hansson *et al* 1991, Hansson 1994, Hansson 1993, Wick *et al* 1995, Watanabe *et al* 1995). Many other authors are still cautious regarding the extent that the immune system is involved in atherogenesis however. Hansson (1994) reminds us that "The presence of blood-borne cells such as monocyte derived mØs and T lymphocytes in vascular tissue does not necessarily imply that an active immune or inflammatory response is occurring. Instead, the cells may be trapped in a forming plaque composed of loose connective tissue elements and thrombi". Further, damaged ECM proteins, especially fibronectin and laminin, leads to increased secretion of TNFα by both T cells and macrophage in the absence of antigen, This can lead to the non-specific attraction of more leukocytes (Hershkoviz *et al* 1993). While it is likely that the initiation of the atherosclerotic process does not rely on the immune system, immune mechanisms likely affect the progression of the disease (Hansson 1993, Libby and Hansson 1991). A great deal of work must be done to clarify what the role of cell mediated immunity is in the atherogenic process since the study of the immunological process in plaque formation is still in the early stages.

Plaque derived T cells are heterogeneous. This likely means that only small numbers of T cells respond to local antigen (Stemme *et al* 1991). Hence, most T cells are probably attracted through immunologically non-specific mechanisms (Blum and Miller 1996); the conditions that exist during an inflammatory response (Hansson 1994).

T cells in plaque exhibit similar receptor expression to that of T cells in patients with other inflammatory conditions such as rheumatoid arthritis (Hansson *et al* 1989), diabetes mellitus (Botazzo *et al* 1985), multiple sclerosis (Hafler and Weiner 1987), and thyroiditis (Katzin *et al* 1989). We know that

plaque derived lymphocytes are almost exclusively $CD3^+$ and that many exhibit characteristics of activation. Approximately one-third of T-cells in atherosclerotic plaque express the activation markers HLA-DR, and very late activation antigen 1 (VLA-1), while lower numbers of cells express the interleukin-2 (IL-2) receptor. This pattern of expression of activation markers suggests that the T cells are activated in atherosclerotic plaque (Hansson *et al* 1989, van der Wal *et al* 1989, Stemme *et al* 1992).

The presence of activated T lymphocytes in plaque has lead Jang *et al* (1993) to state that T cells may have a pivotal role in the atherosclerotic process, possibly by assisting in the development of foam cells. Should this be true, an as yet unproven antigen (or antigens) must be required for atherogenesis. Autoantibodies, known risk factors for coronary artery disease development, are present in plaque which lends support to this hypothesis (Cerilli *et al* 1985, Hansson *et al* 1989). One possible atherogenic antigen is oxidized lipoprotein (Stemme *et al* 1995), which is a potent activator of T lymphocytes in atherosclerosis (Frostegard *et al* 1992). Viral or bacterial proteins are other possibilities.

I.IIId. Smooth Muscle Cells

Vascular smooth muscle cells are the cells that comprise the majority of the medial layer of the arterial wall and maintain vascular tone by contracting during non-systolic periods (*ie* the period when the heart is not propelling blood through the body). Individual SMCs can migrate into the intima, proliferate at what appears to be a low rate (Gordon *et al* 1990), and provide the only source of the connective tissue that is indicative of advanced fibrous plaque (Raines and Ross 1993, Ross 1993). This is atypical in comparison with acute inflammatory responses in other tissues, where fibroblasts normally produce the fibrous tissue.

SMCs are normally MHC class II negative but during atherogenesis, expression of MHC Class II markers occurs. Most cells in plaque are HLA-DR positive, with the greatest proportion being mØ cells, and the next largest proportion being SMCs, where approximately 1/3 of the SMCs were HLA-DR and HLA-DQ positive (Hansson *et al* 1989). This reflects an activation of SMCs in plaque.

Decay accelerating factor is expressed by SMCs in atherosclerotic lesions but is not expressed in the normal arteries. This factor normally protects cells from complement mediated lysis by inhibiting C3/C5 convertase formation which suggests that changes in SMC phenotypes can involve complement regulatory molecules (Seifert and Hansson 1989).

Changes in SMC phenotype are apparent in plaque. In a normal artery, SMCs are arranged in concentric layers, with minimal cytoplasmic endoplasmic reticulum and golgi apparatus, and substantial myofilament numbers. In plaque however, SMCs may take on a synthetic phenotype with a dominant endoplasmic reticulum and golgi apparatus. This allows the cells to devote their activity to the secretion of extracellular matrix (ECM) components and other proteins (reviewed by Raines and Ross 1993).

As mentioned earlier, many researchers accept the Ross "Response to Injury" hypothesis which states, in part, that an endothelial or SMC injury must occur for the initiation of atherogenesis. Therefore, SMC proliferation has long been viewed to be an undesirable contributor to the atherosclerotic process. Weissberg *et al* (1996) have challenged this view. Since SMCs contribute to the stability of plaque through the production of connective tissue in fibrous plaque (Davies 1996, Weissberg 1996, Ross 1993, Libby 1995), Weissberg *et al* (1996) believe that vascular SMCs prevent and suppress acute coronary syndromes. Hence, SMC proliferation may be essential for the development of stable atherosclerotic plaque and clinically may be beneficial.

I.IV. FACTORS THAT ASSIST IN INFLAMMATORY ACTIVATION

In atherosclerosis there are many different growth factors, cytokines, lipids, and other molecules such as free radicals that are essential for the development of atherosclerotic plaque. It is probable that not one of these factors works alone. Rather, they interact with cells and other inflammatory factors to produce a complex network of interactions (Ross 1993, Blum and Miller 1996). The roles of individual molecules have been reviewed by several individuals (Ross 1993, Jang *et al* 1993, Hansson *et al* 1989, Libby and Hansson 1991, Libby *et al* 1995, Blum and Miller 1996). I will endeavor to briefly discuss only the best defined of these factors.

Immunoglobin deposits have been found in atherosclerotic plaques that have not been found in non-atherosclerotic arterial tissue (Hollander *et al* 1979, Hansson *et al* 1984). In cholesterol-fed rabbits, deposits have been discovered where IgG was found in association with collagen fibrils, cytoskeletal fragments, and the cell surface of mononuclear cells (Hansson *et al* 1979; Hansson *et al* 1980). The specificity of these antibodies is unknown however.

Complement factors have also been discovered in plaque with a similar tissue distribution to the IgG antibodies (Hollander *et al* 1979, Hansson *et al* 1984, Pang 1979). When an anti-vimentin IgG antibody binds to vimentin, classical complement pathway activation can occur (Hansson *et al* 1987) which results in the production of large volumes of the C3a and C5a anaphylotoxins which are potent monocyte chemoattractants (Seifert and Kazatchkine 1988). In fact, complement proteins have known relationships with several monocytic and macrophagic functions including: cell spreading, inhibition of migration, stimulation of oxygen metabolism and arachidonate metabolism, chemotaxis, up-regulation of adhesion promoting membrane proteins, and induction of interleukin-1 (IL-1) release (reviewed by Seifert and Kazatchkine 1988). Seifert *et al* (1989) demonstrated that in cholesterol-fed rabbits, local complement activation occurred prior to fatty streak

development. They felt that this supported the idea that cholesterol deposits can activate complement, and that the complement products can serve as chemotactic stimuli for the influx of monocytes into a lesion.

TNF, IL-2, IL-1, interferon gamma (γIFN), and several colony stimulating factors are all important in the inflammation driven response to injury (Ross 1993). Activated mØs in atherosclerotic lesions release IL-1 and TNF after oxidized low density lipoprotein stimulation (Rosenfeld *et al* 1990, Steinbrecher *et al* 1990). IL-1 and platelet derived growth factor (PDGF) are both major growth promoting factors while transforming growth factor-ß (TGF-ß) and γIFN are both growth inhibitors (Hansson 1994).

The IL-1 cytokine was originally identified as a lymphocyte activating factor that was derived from monocytes. It has two forms, Il-1α and Il-1ß. Both induce inflammation and tissue catabolism (Dinarello 1985; Oppenheim 1985; Oppenheim *et al* 1985). Addition of this cytokine to either cultured or *in situ* endothelial cells can cause several effects including: a reorganization of endothelial monolayers (Montesano *et al* 1985), procoagulent activity induction (Bevilacqua *et al* 1984), cell proliferation (Ooi *et al* 1983), and increased vascular permeability (Martin *et al* 1988). IL-1 also promotes leukocyte adhesion through the up-regulation of adhesive receptors for leukocytes on endothelial cells (Bevilacqua *et al* 1985, Cavender *et al* 1986), and SMCs (Wang *et al* 1994), by acting in either an autocrine or paracrine fashion. Endothelial cells produce Il-1 under endotoxin stimulation (Libby, Ordovas, Auger, *et al* 1986), and its secretion can be increased by positive feedback (Warner, Auger, and Libby 1987). SMCs also secrete large amounts of this cytokine after appropriate stimulation (Libby, Ordovas, Birinyi, *et al* 1986).

Monocyte derived IL-1, TNF, and TGF-ß all stimulate SMCs to produce PDGF, which can then act in either a paracrine or an autocrine fashion, to autostimulate a SMC or to stimulate neighbouring SMCs. This can lead to both the migration and the proliferation of SMCs (Libby *et al* 1988). However, it is not necessarily a requirement for plaque development (Schwartz *et al*

1995). At very high levels of PDGF, the PDGF receptors on SMCs are down regulated in a typical negative regulatory fashion (Battegay *et al* 1990).

γIFN stimulates HLA-DR expression on cultured rat aortic SMCs and on endothelial cells (Hansson, Holm *et al* 1988). It inhibits the proliferation of (Blum *et al* 1995, Hansson, Jonasson *et al* 1988), and collagen production by (Libby *et al* 1995, Friesel *et al* 1987), SMCs. γIFN expression is enhanced by TNF, which is released by mononuclear cells in fatty streaks. Mononuclear cells from patients with ischemic heart disease release increased levels of both γIFN and TNF upon stimulation by concancavalin-A (Vaddi *et al* 1994).

TGF-ß is produced by the endothelial cells that line the major blood vessels, and by mØs. Active TGF-ß is present in advanced atherosclerotic lesions (Nikol *et al* 1992). It inhibits both SMC (Morisaki *et al* 1991) and epithelial cell (Massague *et al* 1992) proliferation. In SMCs, it stimulates ECM (*ie* collagen) production, up-regulates adhesion receptors, and initiates α-actin synthesis (reviewed by Saltis *et al* 1996). It also stimulates monocyte/mØ chemotaxis. It has been suggested that low levels of TGF-ß (*ie* too low to prevent SMC proliferation) may lead to fatty streak development (Saltis *et al* 1996). Since decreased levels of TGF-ß have been detected in the serum of patients with severe coronary artery disease (Grainger *et al* 1995) however, Weissberg *et al* (1996) have suggested the contrary; SMC proliferation may actually be beneficial by inhibiting the development of unstable plaque.

I.V. CLINICAL BASIS FOR STRATIFICATION OF PATIENTS

A primary objective of this thesis is to analyze the activity of circulating blood monocytes from patients with varying degrees of atherosclerotic development. As unstable plaque appears in patients, a corresponding degree of clinical stability, or instability, will be exhibited. In order to separate those patients with progressive plaque development, from those who have stable plaque development, heart patients were stratified based on their clinical

stability. To cover the basis for clinical stratification of patients in general, however, is well beyond the realm of this thesis. Instead, selected methods for ascertaining the clinical stability of heart patients will be discussed based on their importance in the stratification of heart patients in general, and in the stratification of blood donors for this study.

Once a detailed history has been taken, several laboratory tests can be used to clarify whether or not a heart condition is present and, if one is, what type of heart condition it may be. In general, one uses the simplest and least invasive tests first and progresses to the more complex and invasive procedures as required. Some tests are noninvasive in nature, since they do not involve the insertion of instruments into the body, while those procedures that do require intravascular instrumentation are referred to as invasive tests. Examples of noninvasive tests include: resting electrocardiogram (ECG or EKG), chest X-ray, magnetic resonance imaging (MRI), nuclear imaging (*ie* thallium stress test), and the exercise stress test (EST). Invasive tests include: intravascular ultrasound and cardiac catheterization (coronary angiography). A more detailed discussion of the most important indicators follows.

I.Va. History and Physical Examination

The initial evaluation of potential coronary artery disease is done by taking a patient's history. Typically, there is a history of a constellation of symptoms including angina, with retrosternal pain, that is exacerbated by exercise and relieved by rest. Associated symptoms may include dyspnea (shortness of breath), diaphoresis (sweating), and/or fatigue. In unstable or pre-infarct (pre-heart attack) patients, there is an acceleration of symptoms where chest or arm pain is noted at lower levels of exertion, or even at rest, and at increasing frequency. The history then, is the basis for further referral to the non-invasive and invasive cardiac tests.

Chest pain or discomfort occurs when the narrowing in the lumen of the artery prevents an adequate blood supply from reaching the heart muscle. This inadequate supply of oxygen to the heart tissue is called *myocardial ischemia.* Chest pain can occur when the blood supply is insufficient during exercise, when the demands on the heart are increased, but is adequate at rest. Discomfort eventually occurs at rest with increasing degrees of stenosis. The "pain" that results is called *angina pectoris* or simply angina. Angina can be precipitated by anger or other emotions, cold weather, or after eating a meal. The degree of angina pectoris is graded according to the degree of effort that is required to produce it. It is described internationally using the criteria of the Canadian Cardiovascular Society (Campeau 1975), while the New York Heart Association's, Functional Classification criteria (Criteria Committee of the New York Heart Association 1964) is used to rate the degree of heart failure (Table 1).

I.Vb. Electrocardiogram (ECG or EKG)

The ECG, first developed by Waller (1887), is commonly used to determine if heart damage has occurred, check for arrhythmias, and detect angina during exercise stress testing. Essentially, an ECG is a graphic record of the heart's electrical potentials (Einthoven 1903, Einthoven 1913, Wilson 1952).

While an ECG does not directly measure the voltages in the heart, it does measure changes in the electrical potential of the heart. Essentially, as heart muscle cells depolarize, or repolarize, fluxes of ions produce detectable electrical potentials. By comparing the electrical patterns from different patients, the ECG is used as the standard for detecting arrhythmias. Other important uses include the diagnosis of myocardial infarction, several heart conditions, and ischemia. In patients with heart "injury", one would expect abnormal ST segments or abnormal Q waves, while myocardial ischemia would produce T wave inversions or ST segment depressions. When

CLASS	CANADIAN CARDIOVASCULAR SOCIETY	NEW YORK HEART ASSOCIATION
I	Ordinary physical activity, ie walking or climbing stairs, does not cause angina. Angina appears with strenuous, prolonged, or rapid exertion.	Patients with cardiac disease but without the resulting limitations of physical activity. Ordinary activity does not cause undue fatigue, palpation, dyspnea, or angina.
II	Slight limitation of ordinary activity. Walking or climbing stairs rapidly, walking uphill, walking or stair climbing after meals, in cold in wind, or when under emotional stress, or only during the few hours after awakening, Walking more than two blocks on the level and climbing more than one flight of ordinary stairs at a normal pace and in normal conditions.	Patients with cardiac disease resulting in slight limitation of physical activity. They are comfortable at rest. Ordinary physical activity results in fatigue, palpitation, dyspnea, or anginal pain.
III	Marked limitation of ordinary physical activity. Walking one to two blocks on the level and climbing more than one flight in normal conditions.	Patients with cardiac disease resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary physical activity causes fatigue, palpitation, dyspnea, or anginal pain.
IV	Inability to carry on any physical activity without discomfort-anginal syndrome may be present at rest.	Patients with cardiac disease resulting in inability to carry on any physical activity without discomfort. Symptoms of cardiac insufficiency or of the anginal syndrome may be present even at rest. If any physical activity is undertaken, discomfort is increased.

TABLE 1: The Two Most Common Systems for the Stratification of the Degree of Angina (Canadian Cardiovascular Society) or Heart Failure (New York Heart Association) In Cardiac Patients

combined with the history of a patient, and other laboratory tests, ECGs help provide the basis for judging the stability and prognosis of a patient (Fisch 1988).

The ECG is also used to stratify patients that suffer from prolonged myocardial ischemia into patients that have, or do not have, objective signs of myocardial infarction (MI). Those without objective signs of MI exhibit no ST-T changes or T-wave abnormalities, and do not have elevation of serum cardiac enzymes. Patients with objective signs may develop new Q-waves in the ECG, with or without elevation in serum cardiac enzymes, or develop ST-T or T-wave abnormalities with increased serum cardiac enzymes.

I.Vc. Exercise Stress Test (EST)

The one factor that nearly always presents itself in coronary artery disease is recurrent angina (chest, arm, or jaw pain). When this occurs it is important to determine the basis for this pain. One preferred way to do this is by an exercise treadmill test (ETT) especially when measurement of maximal exercise capacity is tested (Sheffield 1988).

A stress test is used to test how well the heart can handle work. With stenosis (narrowing) of a coronary artery, blood flow can often be adequate at rest, but be inadequate when additional demands are placed on the myocardium. Increased demands on the heart muscle are produced with exercise. A resulting inadequate blood supply results in myocardial ischemia which is detected by the monitoring of the patient that is done during the stress test. One indicator of this ischemia is an ST depression on the ECG.

An ETT is performed by attaching heart monitoring equipment to a patient. The patient is initially told to walk slowly on a treadmill. The walking pace can then be increased, and/or the treadmill can be tilted to produce the effect of going up a small hill. Heart and respiratory rates, blood pressure, ECG, level of fatigue, and symptoms of chest pain are all monitored

during the test (Bruce *et al* 1963). A positive EST indicates that an inadequate blood supply exists through the coronary arteries and is indicative of the both the kind and the level of exercise that is appropriate for a patient. Thus, it is a marker of clinical stability and is important in the evaluation of the prognosis of the patient (Sheffield 1988).

I.Vd. Radionucleotide or Nuclear Stress Test

The thallium or MIBI stress test is a type of nuclear perfusion (blood flow) imaging test which tests the adequacy of blood flow to the heart muscle. It is usually done in conjunction with an exercise stress test on a treadmill or bicycle because the exercise protocols are identical for both sets of tests. Alternatively, exercise may be mimicked by injection of dypyrimidamole. When the patient reaches his or her maximum level of exercise, a small amount of (radioactive) thallium (or technetrum) is injected into the bloodstream. Thallium then enters the cells of the heart muscle based on the relative perfusion rates of blood through the coronary arteries. Cardiac images are recorded with a "gamma camera" which detects the thallium. Portions of the heart muscle that are poorly perfused contain a lower than a normal amount of radionucleotide. Different patterns are interpreted as follows: 1) If perfusion is normal during both exercise and rest, then blood flow to the coronary arteries is deemed to be normal. 2) If a pattern of low perfusion develops with exercise and becomes more constant over time, ischemia is deemed to be present. 3) If a pattern of low perfusion develops and shifts, this is interpreted as the occurrence of a recent myocardial infarction. 4) If no thallium is seen in an area of the heart muscle, it is indicative of a prior myocardial infarction where the cardiomyocytes are dead and the heart tissue has scarified (Zaret and Berger 1990).

I.Ve. Coronary Angiography

Cardiac catheterization with coronary angiography is the most accurate test for determining the presence and severity, of coronary artery disease. It is used to determine the degree of arterial stenosis (narrowing), and the number of vessels that are stenosed, through injection of contrast into the coronary arteries. It is also used to measure the blood pressure and oxygen saturation of the blood. However, it does not determine whether a patient's symptoms result from ischemia, nor does it provide a basis for a prognosis on its own.

Cardiac catheterization involves insertion of a thin plastic tube (a catheter) into an artery in the arm or leg. Catheters are then advanced retrograde to the coronary arteries in the heart. Coronary angiography (also called angiocardiography or coronary arteriography) essentially is a radiographic examination of the diameter of the blood vessels or chambers of the heart. As contrast media (a dye containing iodine) passes through the arteries, the X-rays are unable to pass through to the film. Because this contrast medium fills the entire lumen of the artery under question, the pictures reflect the diameter of the arteries and, upon review, the degree of stenosis can be determined along any part of an artery.

I.V OBJECTIVES AND HYPOTHESES

I.VIa. Objectives

1) To develop an assay for testing the level of activation, specifically the migratory capacity of primary isolates of circulating peripheral blood monocytes, which mimics the environment of plaque.

2) To use this assay to determine whether monocytes from patients with unstable coronary arterial atherosclerotic plaque development have increased levels of cellular activity (as reflected by the rate of migration).

I.VIb. Hypotheses

1) Increased monocyte migration *in vitro* reflects in increase in the overall level of activation of monocytic cells.

2) Patients with unstable coronary syndromes (unstable angina and myocardial infarction) have circulating monocytes that have a higher state of activation which is induced by the ongoing inflammatory responses that are associated with unstable plaque formation.

CHAPTER II - DEVELOPMENT OF THE MIGRATION ASSAY: EARLY RESULTS WITH LEUKEMIC CELL LINES

The ultimate goal of this study is to compare the migratory activity of peripheral blood monocytes from heart patients with varying degrees of atherosclerotic disease. At the time that this work was started, the assay methods that were being used did not accurately reflect what a circulating blood cells would encounter in an artery *in vivo*. Thus, the development of an assay for testing the activity levels of monocytes quickly became a preliminary step to this study.

Increased monocyte chemotaxis has been tied to increased activation of circulating monocytes in patients with type 1 diabetes mellitus (Josefsen *et al* 1994). Others have used migration as a means of testing the level of activation of monocytic cells *in vitro* (Yano *et al* 1995). Others have shown that cytokines that activate cells as determined by arachidonic acid release, occurred synergistically with THP-1 cell migration (Locati *et al* 1994). The assay that was optimized for this thesis essentially is a modification of the Boyden chamber migration assay, so named after the author that developed it (Boyden 1962). This was the first chemotaxis chamber that was used for the *in vitro* study of leukocyte migration. As will soon be discussed, the primary modification which was made to the two chamber assay was the addition of an ultra-thin coating of ECM proteins over the filter barrier. This allowed for the measurement of the innate migratory abilities of the cells to be tested. The basic structure of the Boyden chamber, is a two chamber design with two wells that are separated solely by a single filter (see Figure 1a).

Prior to the development of the Boyden chamber migration system, the test that was generally relied upon was the under agarose migration assay (Clausen 1971, Cutler 1974, Nelson *et al* 1975) where monocytes or neutrophils were placed under an agarose gel, and the distance of migration

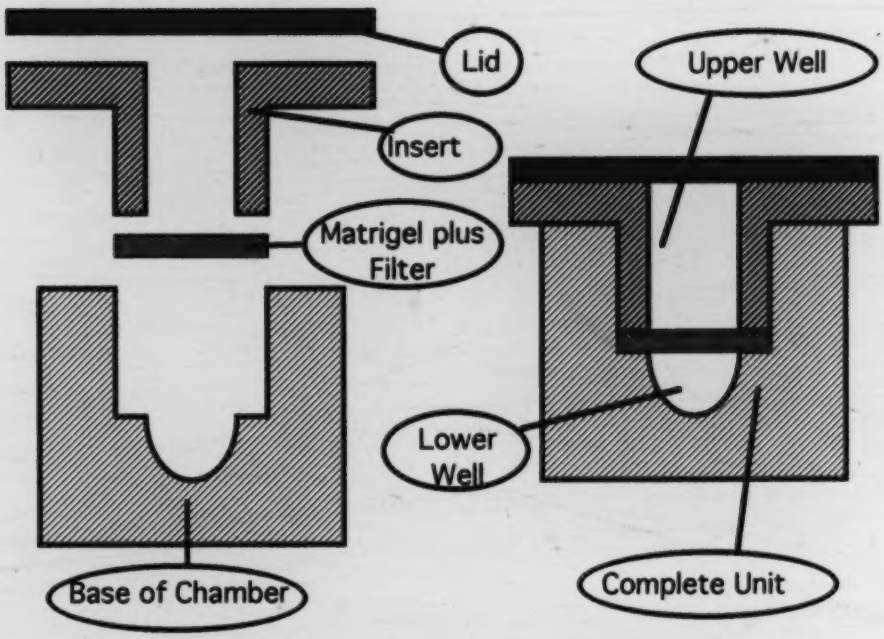

Figure 1a: The basic design of a Boyden Chamber
(shown as a cross section).

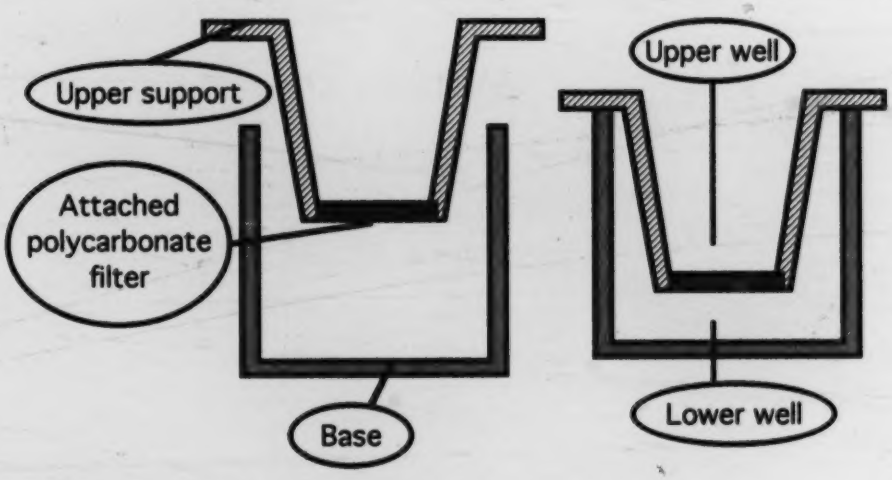

Figure 1b:The basic design of a Transwell Unit
(shown as a cross section)

beneath this gel was measured. However, this assay was quite sensitive to platelet contamination, which often lead to low migration rates (Nielsen 1990). A leading front method has also been used which measured cellular migration distances into a thick filter (Aasen and Schreiner 1986). With both of the older methods however, authors have found reliability problems using human peripheral blood monocytes (Nielsen 1990, Aasen and Shreiner 1986). A detailed comparison of the different migration protocols has not been done with monocytic cells. However, comparisons have been made between the aforementioned protocols using polymorphonuclear leukocytes. In those studies, Boyden chambers demonstrated superior sensitivity over both of the other methods (Kreutzer *et al* 1978, MacFadden *et al* 1985).

Since the original development of the Boyden chamber migration assay, several improvements and modifications have been made to the initial design. Campbell (1977) demonstrated that monocytes could detach from a filter barrier post-migration. Since this study, several different filter compositions have been tested. It was found that polyvinylpyrrolidone-free polycarbonate filters produced better results than regular polycarbonate filters because of a greatly reduced loss of adherent leukocytes post-migration (Harvath *et al* 1980, Gee 1984). New chamber designs have also been developed. Transwell chambers are commercially available which have modifications that allow for multiple sample taking from the lower well without the need to end a test. The basis for this improvement was the incorporation of a sampling port into the experimental design, which eliminated the need for removing the filter prior to sampling the contents of the lower well. In turn, this allowed for improved time course experiments and provided less likelihood of cells from the upper well contaminating the lower well during disassembly of the apparatus prior to sampling. The transwell chambers which were used in these studies comprise one such design (see Figure 1b). Today, both the Boyden chamber and transwell chamber based designs are commonly employed.

One recent major modification to the two chamber migration assay was a coating of the filter with a protein barrier such as Matrigel, an artificial basement membrane. In this way, a uniform barrier was produced over the filters which obstructed the pores and had to be degraded prior to cellular penetration into the lower chamber. The resulting 'invasion assay' was used as a method to study the metastatic potential of various solid tumor cell lines and primary isolates (Albini *et al* 1987, Hendrix *et al* 1987, Repesh 1989, Hendrix *et al* 1990). This was modified further to study the invasiveness of leukemic cells (Janiak *et al* 1994).

The most recent modification of the two chamber migration assay is the first focus of this thesis. It is the production of an assay where, for the first time, Matrigel was used to coat the filter barrier in a migration assay. By presenting cells with the constituents of the ECM instead of a nitrocellulose or polycarbonate surface as in previous two chamber migration assays, the innate rate of leukocyte migration could be measured, for the first time, without the need to add a chemoattractant into the lower well. This chapter will discuss some of the early work that was done in the production of this modified approach to two chamber migration assays; work which relied on the leukemic KG-1 (granulocytic) and THP-1 (monoblastic) cell lines.

II.I. MATERIALS AND METHODS

II.Ia. KG-1 Cell Blindwell Chamber Invasion Experiments

Cellular invasion experiments were performed using the leukemic KG-1 cell line (American Type Culture Collection (ATCC), Rockville, MD, USA) as described elsewhere (Janiak *et al* 1994). This cell line, which was originally isolated from a leukemic child, is granulocytic in nature and represents a leukemic form of the neutrophil. It will spontaneously invade through a Matrigel/polycarbonate filter barrier in an invasion assay (Janiak *et*

al 1994). In such invasion experiments, KG-1 cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM, Gibco BRL, Burlington, Canada) plus 10% Fetal Calf Serum (FCS, Gibco BRL). Cells were grown in unsupplemented IMDM overnight and resuspended in fresh IMDM media immediately prior to use to prevent exogenous addition of cytokines and growth factors. The lower well of a blindwell chamber was filled with 200 µl unsupplemented IMDM. 35 µg Matrigel (Collaborative Biomedical Products, Bedford, MA) in IMDM, was used to coat each 8 micron pore sized polycarbonate filter (Costar/Nucleopore, Toronto, Canada) by drying the mixture overnight. Immediately prior to use, filters were rehydrated for one hour in IMDM media. 200 µl of unsupplemented IMDM was placed in the lower well. The freshly rehydrated filters were then put in place as the barrier between the upper and lower chambers. Units were assembled and 3×10^5 cells in 200 µl media were transferred into the upper well of the blindwell invasion chambers. Assembled units were incubated in a 5% CO_2 incubator for two hours.

Upon completion of the incubation, the media and non-invading cells were aspirated out of the upper well and the filter barrier was carefully removed. Cells that penetrated through the protein/polycarbonate filter barrier, to invade into the lower well, were enumerated by hæmocytometer. Six migration chambers were used for each tested experimental condition. The percent invasion was averaged over all 6 wells by dividing the number of cells that invaded from the upper well, through the protein/filter barrier, and into the lower well, by the number of original cells in the top well (3×10^5). To compare the effects of different concentrations of inhibitor on the KG-1 cellular invasion rates, the percent invasion from each tested condition was compared by dividing the mean percent invasion of the KG-1 cells in the inhibitor containing wells by the mean percent invasion of the KG-1 cells in the control wells. Results were expressed as percent invasion relative to control.

II.Ib. THP-1 Cell Migration Assay

To test the migratory abilities of the monoblastic THP-1 leukemic cell line (ATCC), THP-1 cells were used in the aforementioned KG-1 cell invasion assay and in the migration assay that is described here. In both cases, the THP-1 cells were maintained in DMEM plus 10% FCS until the night prior to use. Before use, the THP-1 cells were placed in unsupplemented DMEM and incubated overnight to lessen any potential effects that growth factors in the calf serum might have on the experiments. The following morning, cells were resuspended in fresh DMEM at 1.5×10^6 cells/ml media. 600 µl of 10^{-7} M N-formyl-methionyl-leucyl-phenylalanine (FMLP), a bacterial peptide with well defined chemotactic characteristics in two chamber migration assays (Nielsen 1990), in DMEM was placed in the lower well of a transwell chamber. 200 µl of THP-1 cell suspension, in the presence of inhibitor or control, was placed in the upper well (3×10^5 THP-1 cells per well). Chambers were incubated at 37° C in a 5% CO_2 incubator for 6 hours. At each time point (1, 2, 4, and 6 hours), 50 µl of media from the lower well was sampled and enumerated. After correcting for the volume that was removed for sampling, the percentage of the cells that migrated from the upper to the lower chamber was calculated at each time point. Since 50 µl of sample was taken at each time point, corrections were done as follows: 600 µl was used as the volume of the lower well at the first time point, 550 µl was used as the volume at the second, 500 µl at the third, and 450 µl at the fourth. Generally 6 wells were used per experimental condition, never less than 4, and the percent migration was averaged over all of the tested wells in each experiment.

II.II. RESULTS

II.IIa. KG-1 Cell Invasion Experiments

Janiak *et al* (1994) previously characterized a leukemic cell invasion assay that relied on a two chamber system with a Matrigel barrier over a polycarbonate filter. From their prior invasion assay studies, we know that when 35 μg of Matrigel is used to coat a filter, and 3×10^5 KG-1 cells are placed in the upper well of the invasion assay apparatus, the innate KG-1 cell invasion rate can be expected to be approximately 2.5% in a 2 hour invasion assay. They further found that benzamidine, a matrix metalloproteinase inhibitor, inhibits this invasive activity (Haroon Hashmi, University of Alberta, personal communication).

After some assistance by Haroon Hashmi in learning the invasion assay technique, I found that, SERP-1, a serine proteinase inhibitor (SERPIN) with known anti-inflammatory (Upton *et al* 1990) and anti-atherosclerotic properties (Lucas *et al* 1996), significantly ($p<0.0001$ by analysis of variance (ANOVA)) decreased the percentage of invading cells (Figure 2). Similar decreases in KG-1 cellular invasion occurred when KG-1 cells were treated with the benzamidine control in association with Haroon Hashmi ($p<0.0001$ by ANOVA). To compare individual conditions to each other, the Fisher PLSD multiple comparison test was used. A non-significant decrease in cellular invasion was found between the control wells and the SERP-1 treated samples at 50 pg of SERP-1 ($p=0.4374$). Differences became significant at each of 100 pg ($p=0.0274$), 200 pg ($p<0.0001$), and 400 pg ($p<0.0026$) doses of SERP-1 (Lundstrom *et al* 1996). The relative percent invasion values for these same samples were (mean ± standard deviation): at 50 pg SERP-1 (1.32 ± 0.22), 100pg SERP-1 (0.81 ± 0.19), 200 pg SERP-1 (0.65 ± 0.40), and 400 pg SERP-1 (2.22 ± 0.40). The control well had an average value of (2.65 ± 1.34) and the benzamidine inhibitor had an average of (0.22 ± 0.1).

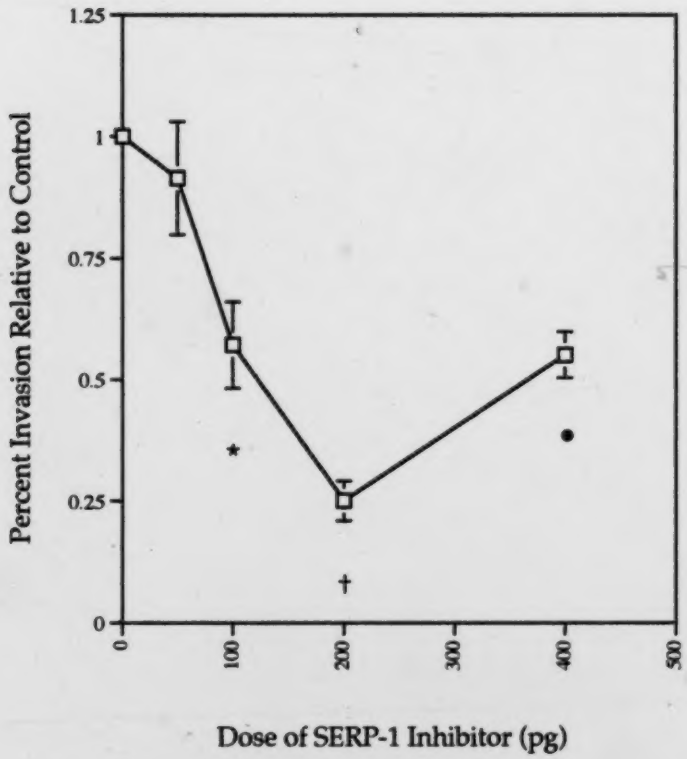

FIGURE 2: The Inhibition of KG-1 Cellular Invasion by the SERPIN, SERP-1. 35μg of Matrigel was dried onto the blindwell chamber filter. Filters were rehydrated prior to use. Cells which invaded into the lower well were counted by hæmocytometer. The percentage of invading cells was calculated by dividing the number of cells in the lower well by the initial number of cells that were added to the upper well (300 000). The relative percent invasion was then determined by dividing the percent invasion for each experimental condition by the percent invasion for the control wells. Each point represents the mean of a minimum of five wells. Error bars represent the Standard Error. (*$p<0.05$, † $p<0.0001$, •$p<0.003$)

II.IIb. THP-1 Cell Migration Experiments

To ascertain whether monocytic cell invasion could occur through a Matrigel barrier, the monoblastic THP-1 cell line was used in the above invasion assay. The results showed that THP-1 cell invasion rates were marginal at best, or too low to be detected. To ensure that the lack of invasion was not due to faulty THP-1 cell activity, THP-1 cell migration through uncoated filters was tested. Under these conditions, no migration occurred in the absence of FMLP chemoattractant. Optimum THP-1 cell migration did occur however, through the uncoated filters in the presence of 10^{-7} M FMLP chemoattractant. FMLP dependent migration into the lower well exhibited a linear increase in cell numbers over a 6 hour time period (Figure 3).

Interestingly, the SERP-1 protein that caused an inhibition of KG-1 cell invasion had no effect on THP-1 cell migration. Differences between these experiments were the types of cells, presence of an ECM protein barrier, and the presence of FMLP chemoattractant. Since the SERP-1 proteinase didn't seem to have a direct effect on cellular activity, results suggest that SERP-1 normally acts on a protein that is important in the degeneration of the ECM barrier instead of directly on the cells themselves (discussed in section III.IIb).

II.III. DISCUSSION

II.IIIa. KG-1 Cell Invasion Experiments

SERP-1 was tested in the existing invasion assay with KG-1 cells to see if it could inhibit cellular invasion. This was done as a precursor to the experiments on primary isolates of circulating monocytes and was performed in parallel with the development of the monocyte migration assay. An early hypothesis was that the SERP-1 proteinase, which was anti-inflammatory (Upton *et al* 1990) and anti-atherogenic (Lucas *et al* 1996), could inhibit

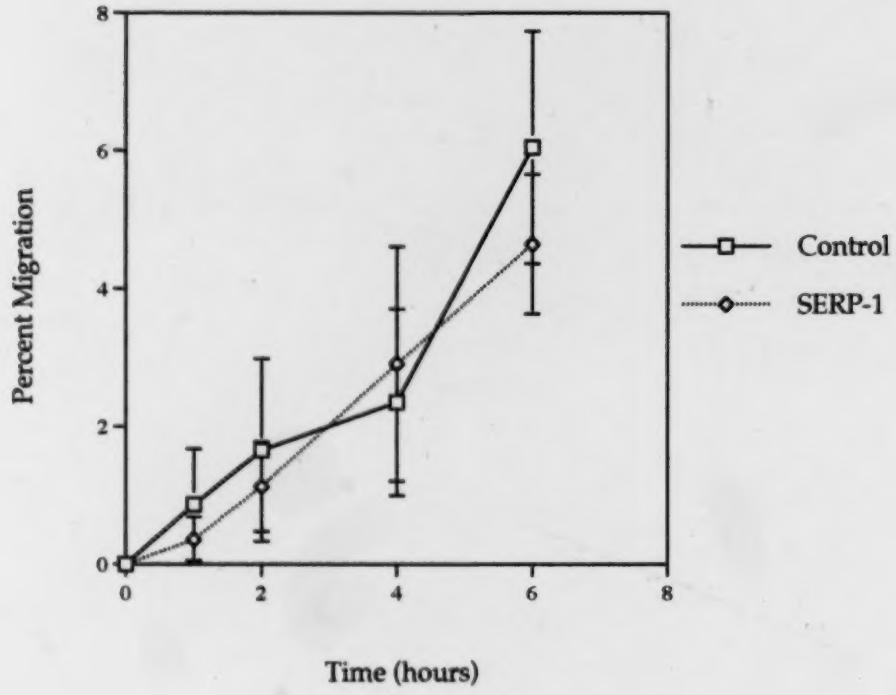

FIGURE 3: Time Course of THP-1 Cell Migration and the Potential Effects of SERP-1 on That Migration Using Uncoated Filters and Chemoattractant. 10^{-7}M FMLP chemoattractant was added to the lower well. Filters were uncoated. Cells that migrated into the lower well were enumerated by hæmocytometer. The number of cells in the lower well was divided by the initial number of cells in the upper well (300 000) to yield percent migration. In the initial calculation of the number of cells in the lower well, cell counts were normalized by correcting for the 50μl volume of media that was removed at each time point (see section II.Ib).

cellular invasion by acting directly on the cell. To see if this occurred, the invasion assay was used to compare the rate of cellular invasion of KG-1 cells with and without SERP-1 treatment. This was also compared to results with an internal control inhibitor of invasion, the metalloproteinase inhibitor benzamidine, and results with THP-1 cells (section II.IIb.). SERP-1 caused a significant decrease in cellular invasion which was comparable to that of the benzamidine control (section II.IIa).

If one assumes that the production of proteinases are tied to cellular activity levels, one could interpret the decrease in KG-1 cellular invasion rates by both benzamidine and SERP-1 as being representative of a decrease in the overall level of activation of the KG-1 cells. While this is theoretically possible, there is a far more likely explanation for the SERP-1 and benzamidine mediated decrease in KG-1 cellular invasion rates. Proteinases, or their inactive proenzymes, that are important for the degradation of Matrigel constituents were likely inhibited directly by both of the tested substances.

The Matrigel barrier is composed of a complex mixture of ECM proteins (Kleinman *et al* 1982, McGuire and Seeds 1989). With the experimental protocol that was used in the invasion assay, a physical protein barrier was produced which inhibited KG-1 cell migration into the lower chamber in the absence of KG-1 cell derived proteinases (Janiak *et al* 1994). These proteinases, which would normally function in the degradation of this protein barrier, could possibly be secreted in inactive form. The SERP-1 protein could then be inhibiting the activation of a matrix metalloproteinase activating enzyme by binding irreversibly to it. In the absence of this inhibitor, matrix metalloproteinase activation would likely normally be followed by cellular penetration through the ECM protein barrier *in vivo*.

Support for the hypothesis, the inhibition of a proteinase activity is responsible for the decrease in KG-1 cell invasion, is enhanced when one considers what is known about the SERP-1 protein. Plasmin, a known activator of matrix metalloproteinases, is inhibited by SERP-1, as are tissue

plasminogen activator and urokinase (Lomas *et al* 1993). Therefore, the most likely explanation for the SERP-1 driven inhibition of KG-1 cell penetration into the lower well, was a titration of the activity of an ECM degrading proteinase. This is supported in a comparative study of the ability of different cells to degrade an ECM protein barrier. Neutrophils predominantly exhibited proteinase activity while monocytes primarily released sulfatase activity (Bartlett *et al* 1995). When one recalls that the SERP-1 protein is a SERPIN, and that the typical activity of a SERPIN is to irreversibly bind to a serine proteinase, thereby deactivating it, this hypothesis appears to be even more likely.

III.IIb. THP-1 Cell Migration Experiments

The inability of THP-1 cells to invade through a Matrigel coating had an interesting correlation with earlier results. Janiak, M. *et al* (1994) have previously shown that under similar experimental conditions, another commonly utilized monocytic cell line (U-937) had marginal invasion rates. Further, as will be discussed in Chapter 3, normal peripheral blood monocytic cell isolates were non-invasive in the invasion assay. Taken together, results strongly suggest that non-stimulated monocytic cells are non-invasive through an ECM protein barrier *in vitro* in 2 chamber invasion assays.

The FMLP chemoattractant is a peptide of bacterial origin that acts as a potent chemoattractant for monocytes, mature mØs, and neutrophils. As discussed earlier, this peptide induced the migration of THP-1 cells through uncoated filters during the migration experiments. This confirmed that the THP-1 cells that were used in the invasion assay were otherwise active by showing that normal cell migration could occur in the absence of the protein barrier. The pattern of migration in these experiments was reflective of the results of previous authors, in prior experiments, where chemoattractants

such as FMLP were required for cell migration (Nielsen and Olesen Larsen 1983, reviewed by Nielsen 1990).

The THP-1 cell migration results were markedly different from the earlier KG-1 cell invasion assay results. *In vivo*, a peripheral blood monocyte would not be expected to be invasive without the appropriate stimulation (*ie* endothelial cell damage). Further, mØs tend not to invade deeply into the arterial tissue during atherogenesis. Instead, they localize immediately below the endothelium of the artery (Faggiotto and Ross 1984, Faggiotto *et al* 1984, Davies *et al* 1988). In a study where endothelial cells, platelets, monocytes, lymphocytes, and neutrophils were tested for their ability to degrade an ECM protein barrier, monocytes had the least ability to degrade the barrier (Bartlett *et al* 1995). Neutrophils, represented here by KG-1 cells, by their nature are far more invasive when activated. A leukemic cell line, such as the KG-1 cell line, could behave atypically from normal primary cell isolates when one considers the cancerous state of these cells. Therefore, while KG-1 cells could be used as a positive control for a cellular invasion assay, or could be used to test various substances that might inhibit the proteinases that are required for ECM penetration, including matrix metalloproteinases, results using KG-1 should not be taken as representative of what would happen in an artery with normal primary blood cell isolates.

When one considers that no Matrigel barrier was present for the THP-1 cells to penetrate through in the migration assay, it was not surprising that migration of the THP-1 cells could not be inhibited by a proteinase inhibitor such as the anti-inflammatory SERPIN, SERP-1. This is because the ECM protein degrading enzymes that were required for penetration of the protein barrier in the invasion assay were not needed in the absence of a protein barrier in the migration assay. When the ability for THP-1 cells to invade through the Matrigel barrier was tested, the THP-1 cells were unable to penetrate through it. Therefore, if the SERP-1 protein acted by inhibiting an ECM degrading proteinase, the ability of a SERPIN to block that activity could

not produce an effect on cell migration *per se*. The THP-1 migration assay results, therefore, complement the prior KG-1 invasion assay results. They provide additional evidence that the most likely cause for the SERP-1 driven inhibition of KG-1 cellular invasion was the inhibition of serine proteinases or matrix metalloproteinases that were important for the degradation of ECM proteins. SERP-1, therefore, did not act by affecting the state of activation of these cells directly.

CHAPTER III - THE DEVELOPMENT AND APPLICATION OF A NEW MONOCYTE MIGRATION ASSAY FOR THE ANALYSIS OF PATIENTS WITH UNSTABLE CORONARY SYNDROMES[2]

Monocytes, and their mature counterpart the mØ, comprise an essential component of the atherogenic process (discussed in Chapter 1). Monocytes are the principle source of the mØs that are present in atherosclerotic plaque. Because of the importance of monocytes in atherogenesis, the role of inflammation in plaque formation, and the role of the monocyte in inflammatory regulation, I tested whether monocyte activity, as expressed by cellular migration assays, was increased in patients with varying degrees of atherosclerotic coronary artery disease. Lymphocyte migration was concurrently examined in this assay using the same cohort of patients.

Results from the earlier leukemic cell studies (Chapter 2) were built upon to develop the migration assay. Peripheral blood monocytes from heart patients and normal donors were used for this purpose. As was the case with the invasion assay, the migration assay relied on a Matrigel coating on the filter. However, in the migration assay, the newly developed coating method did not produce a barrier to migration. Instead, the ECM proteins were part of an ultrathin coating over the surface of the filter which didn't obstruct cellular migration through the pores. Using this migration assay, tests were performed on monocytes from heart patients, and the results were compared to monocytes from normal donors. Results showed a significant increase in monocyte migration in patients with severe ischemic heart disease.

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[2] A version of this chapter has been submitted for publication.

III.I. MATERIALS AND METHODS

III.Ia. Monocyte Isolation

To attain primary monocyte isolates, arterial blood was drawn from 24 patients undergoing cardiac catheterization into two 5 ml ethylenediamine tetra-acetic acid (EDTA) (K_3) vacutainer tubes (Collaborative Biomedical Products) via a femoral arterial or venous sheaths (Cordis) catheter during cardiac catheterization procedures. Additional blood was drawn from 14 normal donors by venous puncture into added vacutainer tubes. The blood which was taken from patients during cardiac catheterization was drawn prior to the administration of contrast media, which is used to block the X-rays during angiography, or any intravenous medications (*ie* heparin, morphine, etc.) which were required during the catheterization procedures.

Peripheral blood mononuclear cells (PBMC) were isolated as soon as possible after blood collection (always within 2 hours) as described by Dai *et al* (1997). The procedure was performed as follows: Blood was diluted 1:1 in phosphate buffered saline pH 7.4 (PBS) and layered over 15 ml Ficoll-Paque Plus (Pharmacia Biotech, Uppsala, Sweden) in a 50 ml polycarbonate conical centrifuge tube (Fisher, Edmonton, AB). The cell suspension was spun at 2200 RPM (880g) in a Sorvall H1000-B rotor in a Sorvall Instruments GLC-4 general laboratory centrifuge at room temperature for 25 minutes with the brake off. Mononuclear cells were collected from the interface, placed in a 15 ml polystyrene conical centrifuge tube (Fisher), washed three times by spinning cells in 15 ml cold PBS at 1850 RPM (720g) for 10 minutes at 4° C. Unless otherwise noted, cells were resuspended in cold DMEM at a concentration of 1.5×10^6 cells per ml and used immediately in the migration assay.

III.Ib. Two-Chamber Monocyte Migration Assay

400µl of unsupplemented DMEM was placed into each lower well. The polycarbonate membranes of the transwell units were coated with 1:50 diluted Matrigel:DMEM and incubated for one hour in a 5% CO_2 incubator at 37° C. Unbound Matrigel:DMEM mixture was aspirated off the filter and the filters were rinsed with PBS. Unless otherwise noted, 3×10^5 PBMCs in 200µl unsupplemented DMEM were added to the upper wells to set up the migration experiments. After the addition of newly isolated PBMCs to the upper chamber, the upper and lower chambers were placed together and, unless otherwise noted, experiments were run for 2 hours at 37° C in a 5% CO_2 incubator. Post-incubation, the media and cells were aspirated out of the upper well, non-migrating adherent cells on the upper surface of the filter were scraped off, and the filters were rinsed several times with PBS.

Unlike the prior invasion assay where almost every cell detached from the filter, large numbers of adherent cells remained attached to the filter in the migration assay. Therefore, adherent cells were enumerated on the bottom of the filter in the migration assay instead of by hæmocytometer in the lower well which is the practice in nearly every two chamber migration or invasion assay. The exceptions involve non-adherent leukemic cells or the measurement of migration by radioactivity. Cells that migrated through the filter barrier and attached to the lower surface of the filter were fixed for 15 minutes in 3% glutaraldehyde (Polysciences, Inc., Warrington, PA). Filters were then incubated for 3 minutes in 0.5% Triton X-100 (ICN Biochemicals, Aurora, OH), stained for 15 minutes in Gill's No. 2 Hematoxylin Solution (Sigma, St. Louis, MO), washed several times in PBS, and given a final quick rinse in deionized H_2O. Filters were air dried overnight and permanently mounted on glass slides in Permount (Sigma).

PBMCs, that migrated through and attached the lower side of the filter barrier, were enumerated based on cellular morphology. Ten randomly

selected high power fields were counted per filter; 3 filters were enumerated for each experimental condition. Comparisons between the different experimental conditions were done by taking the average cell count (*ie* the mean number of cells that were counted over ten high power fields per filter) over each of the three filters that were used per experimental condition.

III.Ic. Confirmation of Monocyte Attachment to the Filter

To confirm that the cells that attached to, and were enumerated on, the bottom of the filter post-migration were monocytes, the migrating cells were stained with anti-CD14 antibody. Since T cells, B cells and monocytes were present in the PBMC mixture that was used in the migration assay, this marker was used to stain the adherent monocytes, with some possible B cell cross-reactivity (see section III.IIId). Filters were also examined by a veterinary pathologist for the morphological characteristics that are associated with monocytes. Experiments were run exactly as described in Section III.Ib above until the glutaraldehyde fixation step. Fixation and staining were done as follows: Adherent cells were fixed to the filter by incubating filters for 30 minutes in 0.1% glutaraldehyde in PBS. Endogenous peroxidase activity was quenched in 0.3% H_2O_2 in methanol for 30 minutes. Filters were washed three times, 5 minutes each wash, in PBS and blocking was done for 15 minutes in 10% normal horse serum in PBS.

Anti-CD14 primary mouse monoclonal antibody (Cat # MHDC1400, Caltag Laboratories, Burlingame, CA, USA) was diluted 1/100 in 0.1% Bovine Serum Albumin (BSA) in PBS and filters were incubated at 4° C overnight in 70 µl of diluted antibody solution. For a negative control, a mouse monoclonal anti-isotypic anti-rabbit IgG antibodies (Sigma) were diluted 1/100 in 0.1% BSA in PBS. By using this type of negative control, the chance of a false positive reaction, by such causes as non-specific binding, was greatly reduced. A second antibody deficient negative control was also used. Filters were washed 3 times in PBS/0.1% BSA, 10 minutes each wash. Biotinylated

secondary antibody (Cat # F0224, Vector Laboratories Inc., Burlingame, CA, USA) was diluted 1/50 in PBS/0.1% BSA and incubated overnight at 4° C. Filters were washed 3 times, 5 minutes each wash, in PBS.

The Vectastain Elite (#PK-6100, Vector Laboratories, Burlingame, CA, USA) avidin-biotin complex (ABC) amplification method was prepared according to the manufacturer's protocol. Filters were incubated for one hour, at room temperature, in the presence of the ABC mixture. Filters were washed 3 times in PBS, 5 minutes each wash, and the filters were cut out of the migration chamber and mounted on glass slides (Fisher). Filters were flooded with liquid 3-3'-Diaminobenzidine (DAB) reagent (substrate) and colour development was allowed to progress until optimal levels were attained (approximately 5 minutes). The remaining liquid DAB was aspirated off and membranes were rinsed several times in PBS. Cells were counter stained for 30 seconds in Gill's No. 2 Hematoxylin Solution, rinsed in PBS, and filters were permanently mounted on glass slides in Permount and viewed by light microscopy.

III.Id. Transmission Electron Microscopy

After I coated polycarbonate membranes with 1:50 diluted Matrigel: DMEM mixture for 1 hour in a 5% CO_2 incubator, aspirated off the unbound mixture, and rinsed with PBS, pH 7.4, Dr. Marita Hobman fixed the filters with 2% glutaraldehyde and 3% paraformaldehyde in 0.1% cacodylate-HCl buffer, pH 7.2, at room temperature for 1 hour. Filters were postfixed in 1% OsO_4 for 1 hour, stained with 2% uranyl acetate, pH 6.0, dehydrated in graded ethanols, and embedded in Epon. Honey Chan cut ultrathin sections (cut perpendicular to the membrane) which were placed on nickel grids and on nickel grids were stained with uranyl acetate and lead citrate. We viewed the filters together in a Philips 4-10 electron microscope. In further studies, a filter was coated and stained as outlined above by the Pathology Department

of the London Health Sciences Centre. Further electron micrographs were taken of the porous region of the filters.

III.Ie. Patient Selection and Stratification

Patients who were admitted for elective or urgent cardiac catheterization were considered as potential study candidates. Each patient was stratified according to the: a) degree of coronary stenosis, b) degree of angina, c) urgency of intervention, d) presence of recent myocardial infarction, and e) by MIBI scan or ETT stress tests (see Table 2 for detailed classification). Cardiologists who were blinded to the results of the monocyte migration assays were used to judge the degree of coronary artery stenosis, MIBI scans, and ETT stress test results.

Patients were assigned a numeric value according to the severity of disease that was present for each marker of clinical instability (see Table 2). Donors that were rated as normal under a given category, scored a "1" in that category, while donors that exhibited moderate severity in a given category scored a "2", and patients who exhibited severe symptoms scored a "3". In order to obtain a final stratification for clinical stability, scores for: degree of angina, urgency of intervention, myocardial infarction, and stress test/MIBI scan categories were totaled and ranked. A score of 11 or 12 was deemed severe, 6-10 moderate, and 4 or 5 normal or mild. The degree of coronary stenosis was judged independently based on the coronary angiogram results.

Patients were placed into a severe ischemic heart disease (IHD) category overall if they exhibited combined severe clinical instability and severe coronary stenosis. Patients were placed in the moderate IHD category if they had either severe stenosis or severe clinical instability, but not both, or if they scored moderate in each category. Donors were rated as normal if they were normal both in terms of coronary stenosis and in clinical stability.

PATIENT CRITERIA	DEGREE OF ANGINA	URGENCY OF INTERVENTION	MYOCARDIAL INFARCTION	STRESS TESTS	DEGREE OF CAD
NORMAL CONTROL OR LOW RISK	no chest pain or chest pain only with extreme exertion	angioplasty or CABG not done for a period greater than one month post diagnostics	not present within 2 weeks prior to blood collection	normal MIBI and/or an ETT lasting longer than 9 minutes without chest pain or ΔST. Or, the test was not done because the donor was healthy	less than 70% stenosis in all vessels
MODERATE CAD	chest pain with moderate activity	angioplasty or CABG performed within 1 month of diagnostic testing	recent (within 2 weeks of blood collection)	MIBI showed small area of ischemia and/or an ETT lasted more than 6 min with ΔST	at least 70% stenosis in a minimum of 2 coronary artery vessels
SEVERE CAD	a history of nocturnal chest pain or chest pain at rest	angioplasty or CABG performed within 1 week of diagnostics	recent with small or nonexistant Q wave	MIBI showed a moderate to large area of ischemia and/or an ETT lasted less than 6 min with ΔST and/or chest pain	atleast 90% stenosis in 2 coronary artery vessels

TABLE 2: Criteria for Stratifiing Donors into Normal, Moderate Ischemic Heart Disease (IHD), and Severe IHD Categories. (CABG = coronary artery bypass grafting, MI = myocardial infarction, ΔST = electrocardiogram changes in the S-T segment during an exercise stress test, ETT = exercise treadmill test).

III.If. Statistics

The three categories of donors (*ie* severe, moderate, and normal) were compared by ANOVA and the Fisher protected least significant difference (PLSD) multiple comparison test using a significance level of 1%. The potential effects of each of the heart medications on monocyte migration was tested for by two-tailed, unpaired T tests. Migration rates from each of the categories were compared against the presence of each of the heart medications that patients were prescribed. Differences were considered significant when $p<0.01$.

This study was reviewed and approved by the University of Alberta Hospital's Ethics Committee.

III.II. RESULTS

III.IIa. Peripheral Blood Monocyte Isolation Procedure Development

The use of primary cell isolates proved to be far more difficult than the use of cell lines. In an attempt to attain good yields of highly purified monocytes, two density-gradient centrifugation monocyte isolation procedures were attempted. Different Percoll gradients were attempted according to methods that were published by several authors, along with modifications of those methods (Gmelig-Meyling and Waldmann 1980, Hardin and Downs 1980) but cell yields were always too low for use in the migration assay. Therefore, the Ficoll-Paque, density centrifugation based, purification procedure was used to isolate the PBMCs as is outlined in section III.Ia.

During this isolation procedure, there was an absolute requirement for maintaining the PBMCs at 4° C. Failure to keep the PBMCs in the cold lead to a loss of the monocytes, most likely by adherence of these cells to the sides of the centrifuge tubes during the washing steps. Similarly, the tubes that held

the cells prior to the experiments needed to be kept on ice or the monocytes would also be lost. Siliconization of the tubes did not prevent monocyte loss at room temperature. Lymphocytic cells remained in suspension however.

III.IIb. Development of the Monocyte Migration Assay:

PBMCs from normal donors, and patients with IHD, were used to develop a two chamber monocyte migration assay. A 1:50 dilution of Matrigel:DMEM yielded a desirable protein coating for the comparison of cellular migration. With low dilutions of Matrigel:DMEM, monocytes did not penetrate through the protein barrier. At the 1:50 dilution however, significant monocyte could occur because of the less than 0.1 μm thick (Figure 4A), ECM protein coating on the upper surface of the filter and along the inside of the pore (Figure 4B). A few pores were obstructed at the 1:50 dilution but the majority provided no barrier to migration. At this concentration, the ECM coating allowed for detectable monocyte migration over the ECM proteins, through the membrane pores, and onto the underside of the filter.

Experiments in two chamber migration assays have always relied on the addition of a chemotactic agent for monocyte migration to occur. This gradient is normally produced by adding a chemoattractant such as FMLP into the lower well. Interestingly, with the ECM protein coating on the upper surface of the polycarbonate filter, PBMCs migrated without the addition of a chemoattractant into the lower well. The migration pattern markedly contrasted with what normally occurred in a Boyden chamber in the absence of an ECM protein coating, where cells did not migrate in the absence of the addition of a chemoattractant into the lower chamber.

During optimization experiments time intervals were selected from early and middle time periods. These time course experiments demonstrated

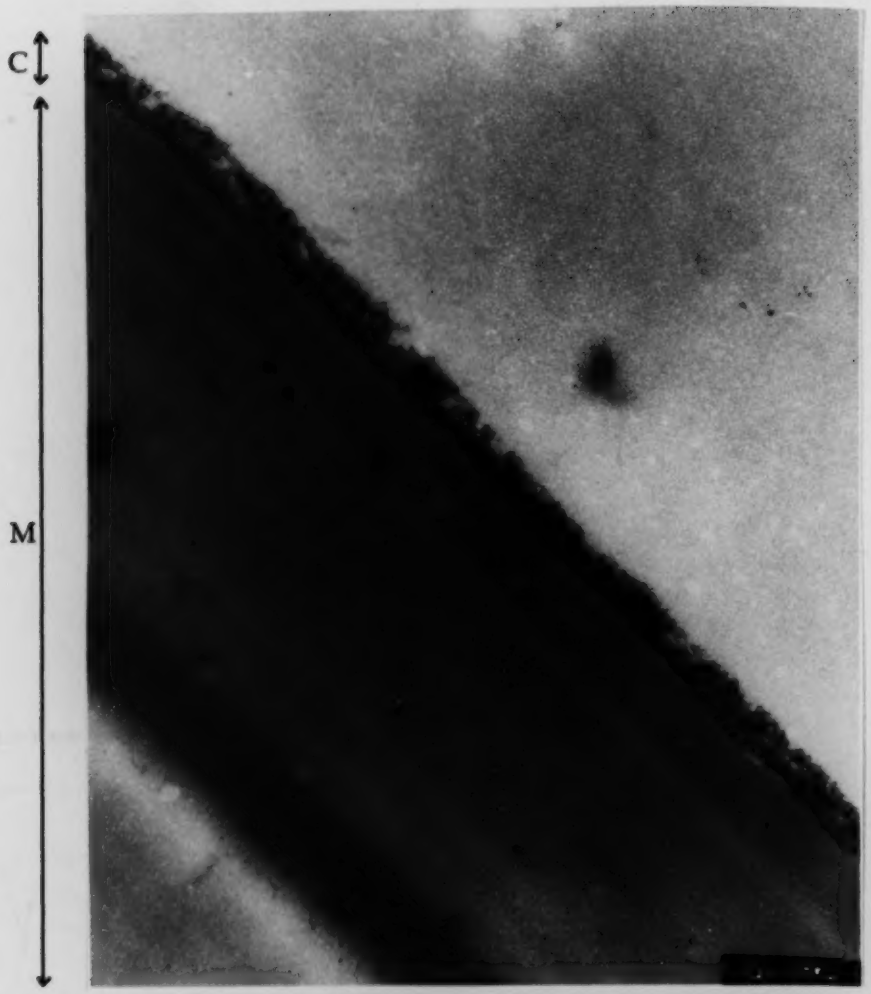

Figure 4A: Transmission Electron Micrograph of the ECM coating. The 40 000 X magnified micrograph was taken over a non-porous section of membrane. Filters were coated in identical fashion to the filters that were used in the migration assay with a 1:50 dilution of Matrigel:DMEM, a 1 hour incubation in a 5% CO_2 incubator, aspiration of unbound protein mixture post-incubation, and a PBS rinse of the coated membrane. Filters were cut perpendicular to the surface of the membrane to produce the cross section of the filter which is shown here. (C = ECM protein coating, M = polycarbonate membrane.)

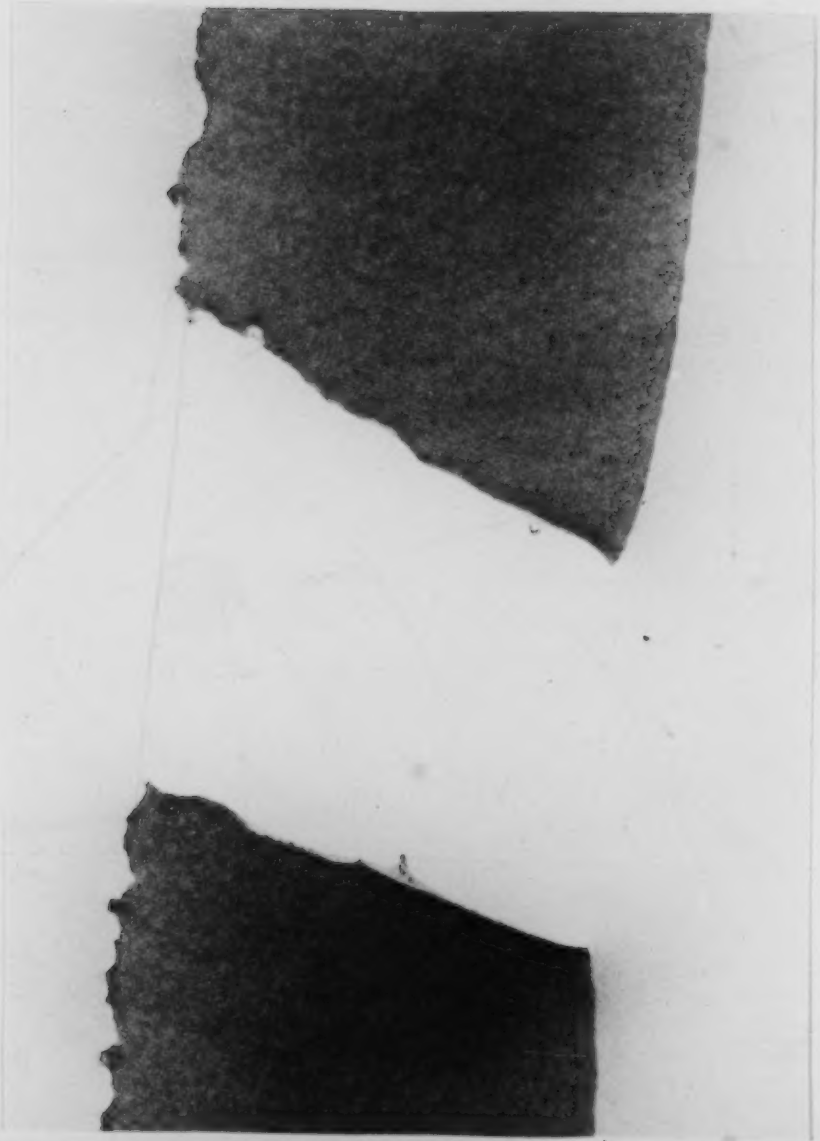

Figure 4B: Transmission Electron Micrograph of the ECM coating. Filters were treated as outlined in Figure 4A. The micrograph is shown magnified 10 000 X and is centred on a filter pore. The dark grey area is the polycarbonate filter. The very thin, light-grey shadow, which is particularly apparent on the inner surface of the pore, is the ECM protein coating.

that the maximum number of migrating monocytes could be detected at the 2 hour time interval, the time interval that was used in the migration assay for the comparison of monocytes from different patient populations. A time-course experiment, using early time periods, showed a linear increase in cell numbers from 12 minutes through to one hour. Cell numbers increased at a lower rate until attaining the maximum migration level at 2 hours (Figure 5).

Nielsen and Olesen Larson (1983), and Campbell (1977) showed, in prior Boyden chamber migration assays, that a linear increase in the number of migrating cells occurred as the number of cells that were introduced into the upper well were increased. The same pattern appeared in this migration assay as the number of cells which were added to the upper well were increased (Figure 6). After a critical number of cells were reached, migration increased in proportion with the number of cells that were placed in the upper well.

III.IIc. Use of the Migration Assay to Test PBMCs From Different Patients With Atherosclerotic Disease

With the optimized migration assay, PBMCs were tested for an increased level of activation cardiac patients versus normal controls. PBMCs were tested from normal donors, patients with moderate or severe IHD, and patients with other inflammatory conditions. Monocytes comprised from 15-30%, generally 20-25%, of the total number of PBMCs in every experiment based on Wright staining of smears of PBMCs on glass slides. Numbers reflect what other authors have found using similar density gradient isolation protocols (Campbell 1977, Böyum 1968, Nielsen *et al* 1981).

Significant ($p<0.0001$ by ANOVA) differences in migration rates were found between the numbers of migrating monocytoid cells from the different categories of donors (Table 2). A small, non-significant ($p=0.1235$ by ANOVA) increase in migration was found between lymphocytoid cells from normal donors and patients with severe IHD, and between donors with moderate

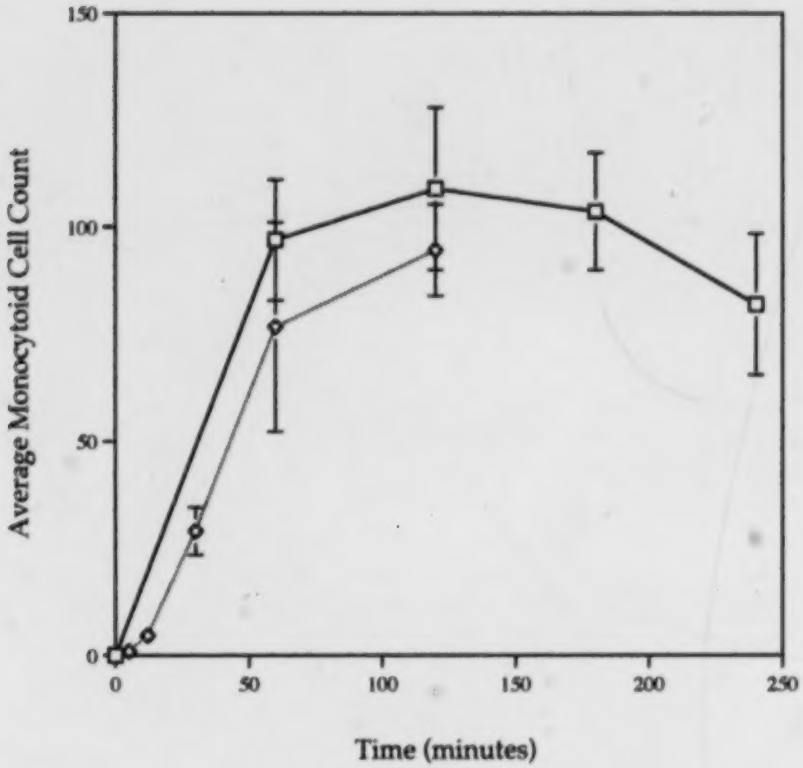

Figure 5: The Relationship Between Monocytoid Cell Migration and Time. Two separate experiments were run using donors with moderate IHD. In each experiment, 300 000 PBMCs were placed in the upper well. Post-incubation, monocytoid cells were enumerated based on cellular morphology with Hematoxylin staining. In the later timecourse experiment, times were selected from 1-4 hours to determine the optimum time for detection of migration. In the early timecourse experiment, 5, 12, 30, 60, and 120 minute intervals were chosen to determine the pattern of migration until the optimal level was reached. Each point represents the mean monocytoid cell count of a total of 10 high power fields per filter, and three filters per time point. Bars represent standard error.
(—□— late timecourse ·····◇····· early timecourse)

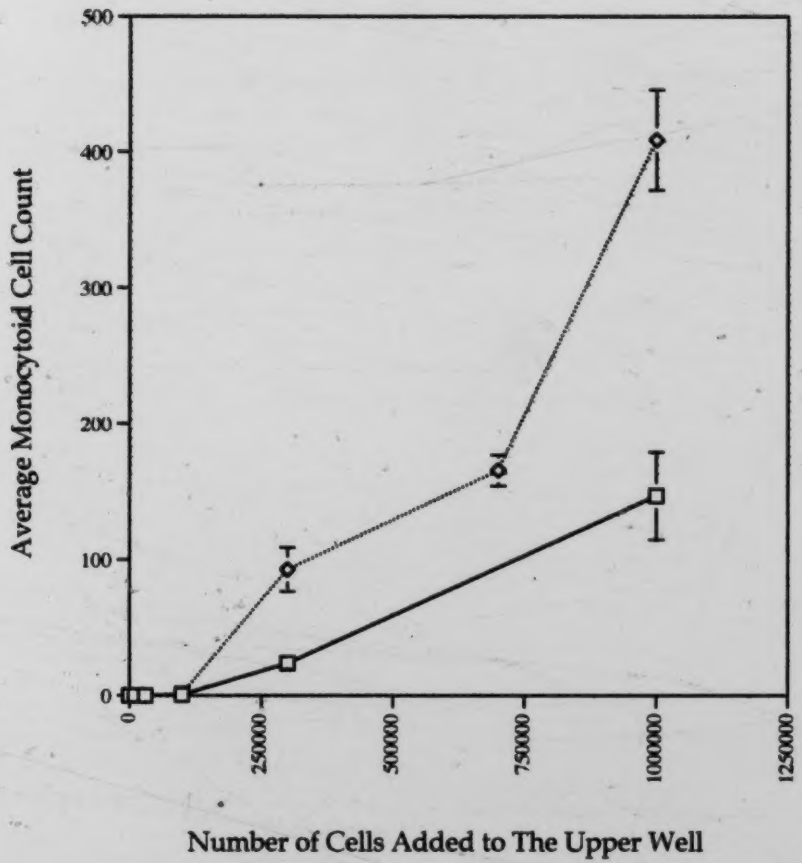

FIGURE 6: The Relationship Between the Number of PBMCs in the Upper Well and the Number of Migrating Cells. Two experiments were run using PBMCs from one normal donor and from one donor with moderate IHD. Experiments were run for 2 hours. Post-incubation, cells on the upper surface were scraped off and cells on the lower surface were stained with hematoxylin. Monocytoid cells were enumerated over 10 high power fields per filter. Three filters were counted and averaged for each of the initial numbers of PBMCs. Where standard error bars are not shown, they were too small to be viewed by the graphing program.
(—□— normal donor ····◇···· moderate IHD)

IHD and severe IHD. There was no difference in lymphocyte migration between the moderate IHD and severe IHD cells. (Figure 7).

The differences in monocyte migration arose between normal controls, patients with severe IHD, and patients with other inflammatory conditions such as rheumatoid arthritis (Figure 8). As compared to both normal controls (Figure 9a), and patients with moderate IHD (Figure 9b), monocytes from patients with severe IHD (Figure 9c) clearly migrated at significantly higher levels ($p<0.0001$ by Fisher PLSD in both cases). Monocytes from the patient with rheumatoid arthritis (Figure 9d) also migrated at significantly higher levels than monocytes from normal donors ($p=0.0030$), and donors with moderate IHD ($p=0.0042$ by Fisher PLSD).

Monocytes from a vasculopath (a patient with extensive peripheral vascular atherosclerotic disease), who was clinically stable according to our criteria, showed a significant increase in migration compared to normal donors ($P=0.0039$ by Fisher PLSD) and patients with moderate IHD ($p=0.0060$ by Fisher PLSD). In contrast, a patient with end stage cardiomyopathy (clinically very unstable), without severe artery disease according to our criteria, did not show an increase in monocyte migration from normal donors ($p=0.6951$ by Fisher PLSD), or donors with moderate IHD ($p=0.7909$ by Fisher PLSD). Therefore, one may speculate that the differences in migration between the different categories of donors were due to the presence of unstable plaque, not just clinical instability (see section III.IIIc).

No differences were found however, between the monocyte migration rates from normal donors or from patients with moderate IHD ($p=0.7205$ by Fisher PLSD) which demonstrated that the aforementioned earlier increase in monocyte migration was not due to the effect of any heart medication (see section III.IIf), from the treatment of the patients, or from the handling of the blood. This was confirmed by using paired two-tailed T-tests to compare the heart medications that patients were on, against the rates of monocyte

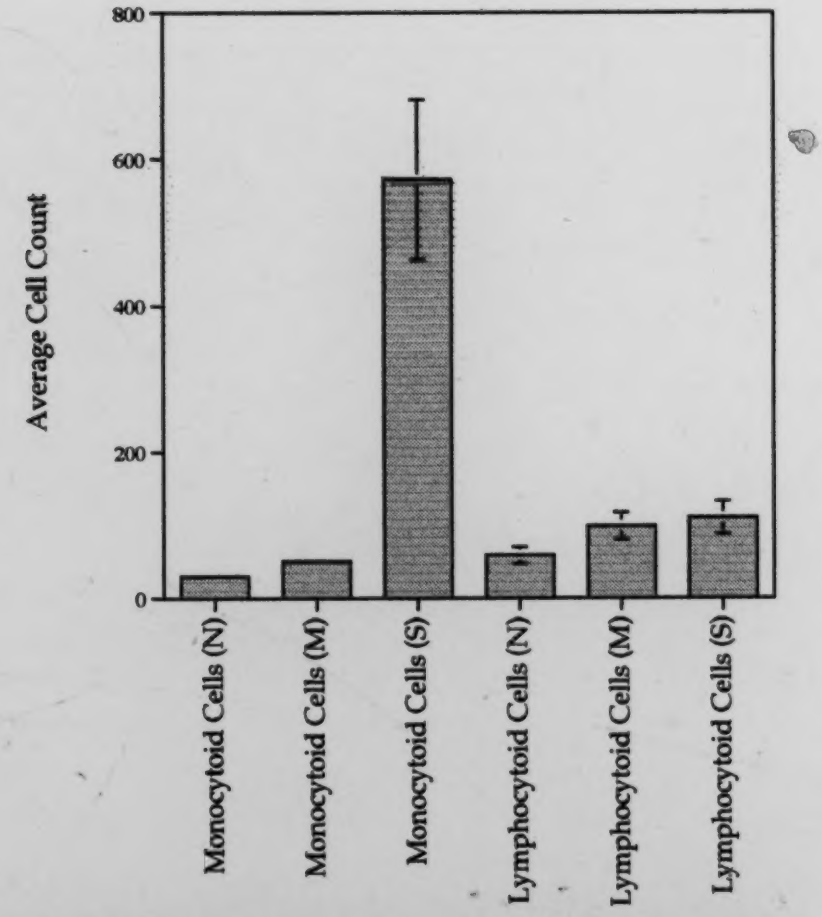

FIGURE 7: Comparison of the Numbers of Migrating PBMCs in the Migration Assay. PBMCs from all migration experiments that used a 1:50 Matrigel to DMEM coating over the filter, 300 000 PBMCs in the upper well, a 2 hour incubation period, and a hematoxylin stain, were subjected to differential counts based on cellular morphology. Three filters were used per experimental condition and averaged. 10 high power fields were counted per filter. Categories describe the class of donor (N= normal, M = moderate IHD or S = severe IHD) and the type of cell (lymphocytoid or monocytoid). Results represent the mean cell counts averaged over all donor samples. Bars represent standard error. When standard error bars are not shown, error was too small to be plotted.

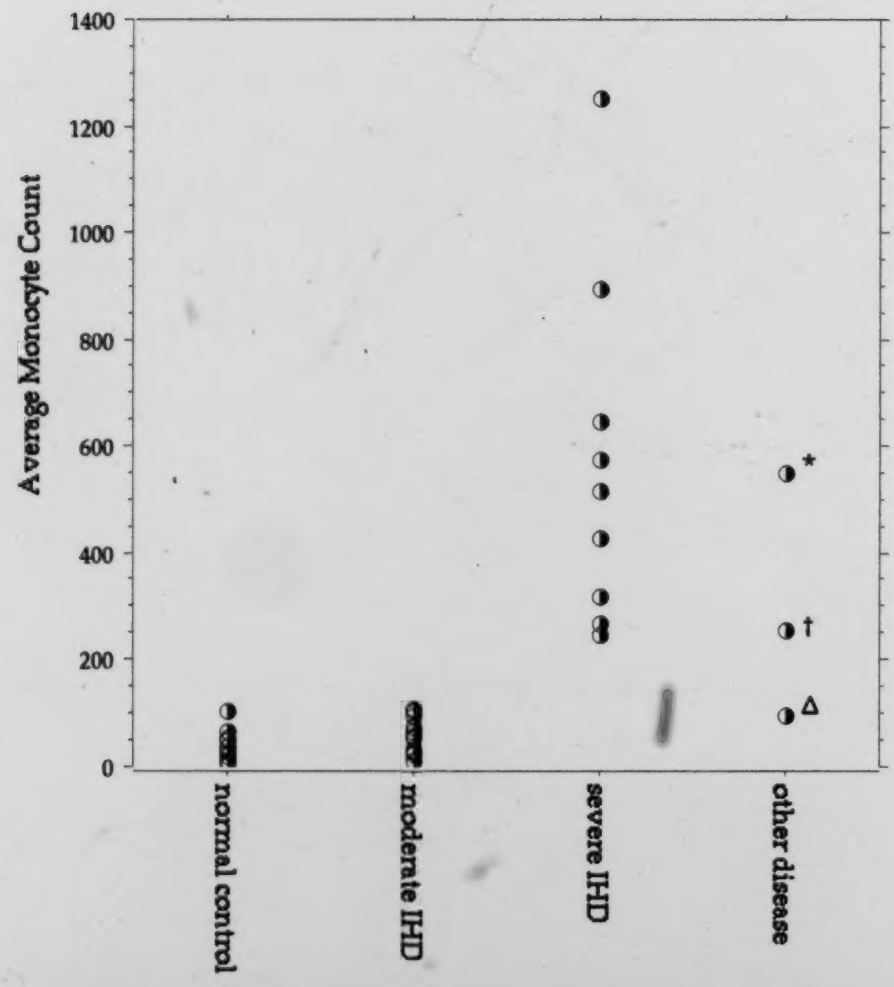

FIGURE 8: Comparison Between The Rates of Monocyte Migration in The Different Categories of Blood Donors. Filters were coated 1:50 in Matrigel:DMEM mixture for 1 hour. 300 000 PBMCs were placed in the upper well and the migration experiments were run for 2 hours. Monocytoid cells were stained with hematoxylin and enumerated based on morphology. Each point represents the mean monocytoid cell count on three filters, from three separate chambers, using PBMCs from a single donor. 10 high power fields were counted per filter. There were 14 normal donors, 15 donors with moderate IHD and 9 donors with severe IHD. (Δ = end stage cardiomyopathy, * = rheumatoid arthritis, † = vasculopath)

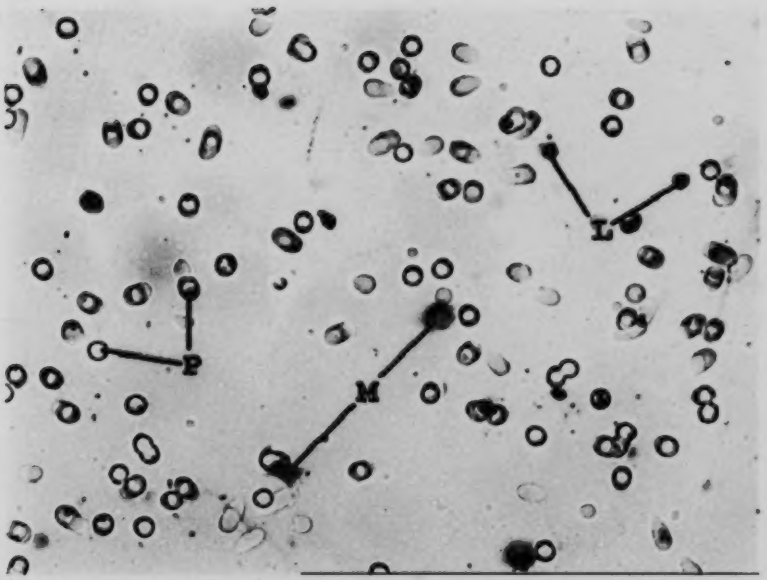

FIGURE 9A: Photomicrograph of Migrating PBMCs on the Lower Surface of the Filter in the Migration Assay. Filters were thin coated with ECM proteins immediately prior to the migration assay. 300 000 PBMCs were placed in the upper well. The chamber was incubated for 2 hours in a 5% CO_2 incubator. Post-incubation, the upper surface of the filter was scraped and the adherent PBMCs on the lower surface of the polycarbonate filter were stained with hematoxylin. A view of the lower surface of the filter is shown with adherent cells. This figure shows the migration of PBMCs from a normal donor. Note the relative sparcity of monocytoid cell migration. (M = monocyte, L = lymphocyte, P = pore)

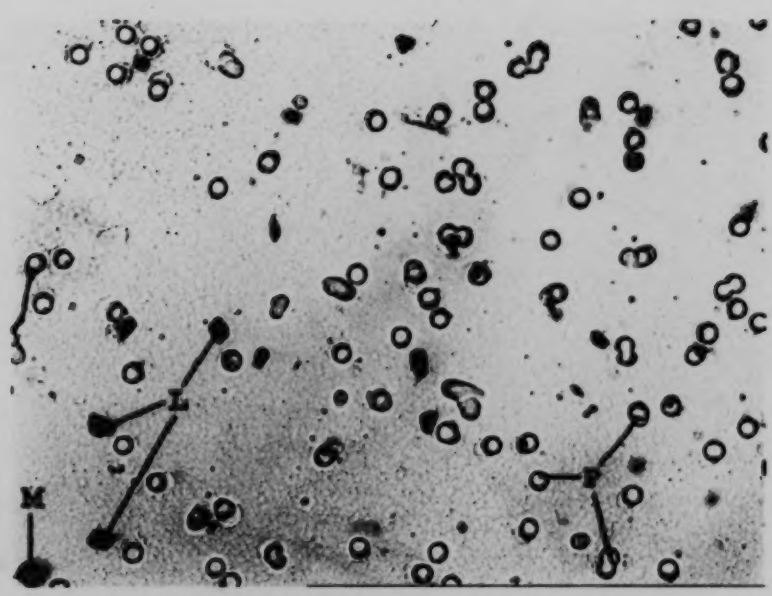

FIGURE 9B: Photomicrograph of Migrating PBMCs on the Lower Surface of the Filter in the Migration Assay. The filter was treated as outline in Figure 9A. This figure shows migration of PBMCs from a donor with moderate IHD. As in Figure 9A, note the relative sparcity of monocytoid cell migration. (L= lymphocyte, P = pore, M = monocyte)

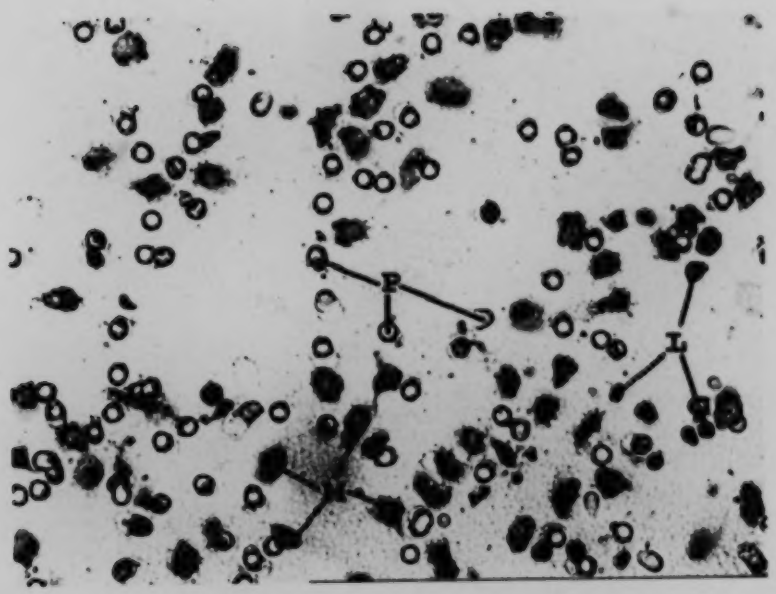

FIGURE 9C: Photomicrograph of Migrating PBMCs on the Lower Surface of the Filter in the Migration Assay. The filter was treated as outline in Figure 9A. This figure shows migration of PBMCs from a donor with severe IHD. Note the increase in the frequency of migrating monocytoid cells with typical horseshoe shaped nuclei. (L= lymphocyte, M = monocyte, P = pore)

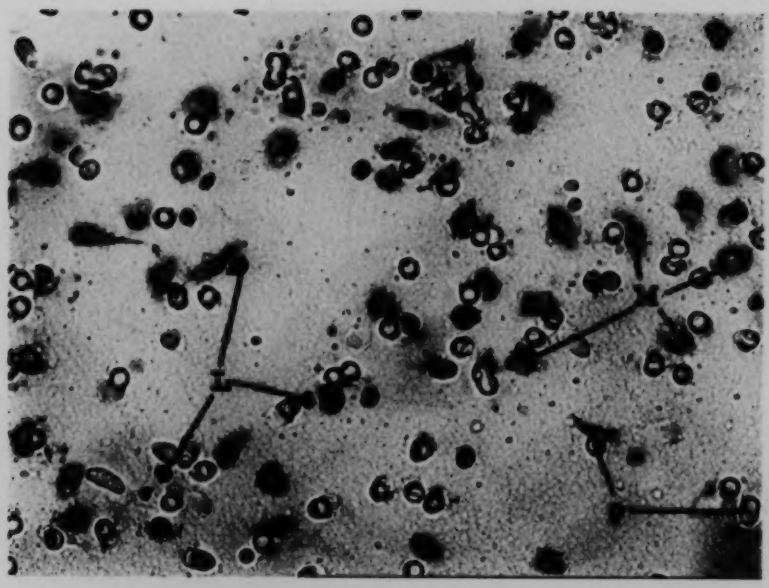

FIGURE 9D: Photomicrograph of Migrating PBMCs on the Lower Surface of the Filter in the Migration Assay. The filter was treated as outlined in Figure 9A. This figure shows the migration of PBMCs from a donor with rheumatoid arthritis. This condition was chosen for comparison of migration rates between different donors because of the importance of inflammation in atherogenesis and in rheumatoid arthritis. As in Figure 9C, note the increase in the frequency of migrating monocytoid cells with typical horseshoe shaped nuclei. (P = pore, M = monocyte, L= lymphocyte)

migration. No significant differences were found in relation to any heart medication or any known underlying disease.

III.IId. Confirmation of Monocyte Attachment and Enumeration on the Filter

Monocytes were initially enumerated in the migration assay based on their cellular morphology. This was accomplished by counting hematoxylin stained monocytoid cells which had horseshoe shaped nuclei and a nucleus to cytoplasm ratio of greater than 1:1. This method is commonly used in cellular migration assays, but it should not be relied on as the sole method of identification since atypically shaped lymphocytes have been reported under different disease conditions (Nielsen 1990) . Therefore, immunostaining was done to confirm morphological staining results using the most commonly utilized monocyte marker, CD14 (Gown *et al* 1986). The CD14 antibody family includes the anti-M3, My4, Mo2 and other antibodies, and together comprise the LPS receptor (Hansson *et al* 1986).

Immunostaining was performed on the cells that migrated through, and attached to, the lower surface of the filters. Immunostaining on these adherent PBMCs was performed directly on the polycarbonate filter using the anti-CD14 antibody. This was done with PBMCs from normal donors and from patients with severe IHD. As can be seen in Figure 10a, of the very few cells that pass through the filter using normal donor blood, the cells with monocytic morphology stain positively with the anti-CD14 antibody, but do not stain with the anti-isotypic anti-rabbit IgG antibody negative control (Figure 10b), nor with a primary antibody deficient control (Figure 10c). Similarly, the migrating monocytoid cells from a donor with severe IHD show positive staining with anti-CD14 antibody post-migration (Figure 11a) while the anti-rabbit IgG antibody negative control (Figure 11b), and primary antibody deficient negative control (Figure 11c), do not demonstrate this staining. In all cases, lymphocytoid cells did not stain positively.

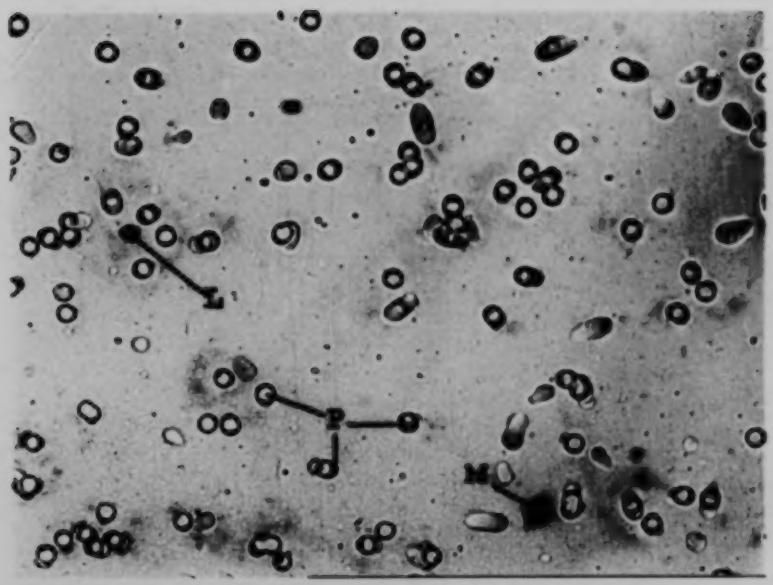

Figure 10A: Immunostain of Migrating PBMCs, From Normal Donors, on the Lower Surface of the Filter in the Migration Assay. The filter was thin coated with ECM proteins for the migration assay. 300 000 PBMCs from a normal donor were placed in the upper well. The chamber was incubated for 2 hours in a 5% CO_2 incubator. Post-incubation, the upper surface of the filter was scraped and the adherent PBMCs on the lower surface of the polycarbonate filter were stained with an anti-CD14 antibody. Cells were counterstained with hematoxylin. Horseradish peroxidase conjugate and DAB substrate were used to develop positively stained cells. A positive reaction is indicated by brown staining with this method. (L = lymphocyte, P = pore, M= monocyte)

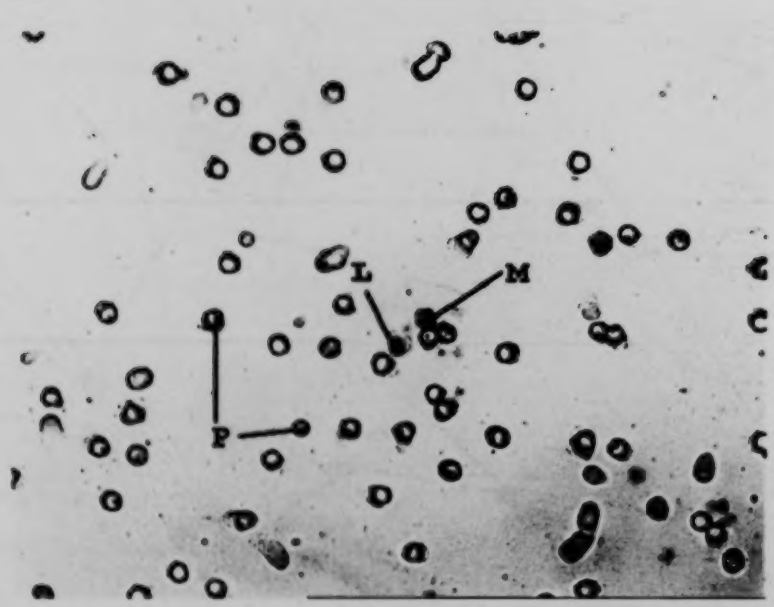

Figure 10B: Immunostain of Migrating PBMCs, From Normal Donors, on the Lower Surface of the Filter in the Migration Assay (Anti-Isotypic Negative Control). The filter was thin coated with ECM proteins for the migration assay. 300 000 PBMCs from a normal donor were placed in the upper well. The chamber was incubated for 2 hours in a 5% CO_2 incubator. Post-incubation, the upper surface of the filter was scraped and the adherent PBMCs on the lower surface of the polycarbonate filter were stained with an anti-isotypic anti-rabbit anti-IgG antibody. Cells were counterstained with hematoxylin. Horseradish peroxidase conjugate and DAB substrate were used to develop positively stained cells. A positive reaction is indicated by brown staining with this method. (P = pore, M= monocyte, L = lymphocyte)

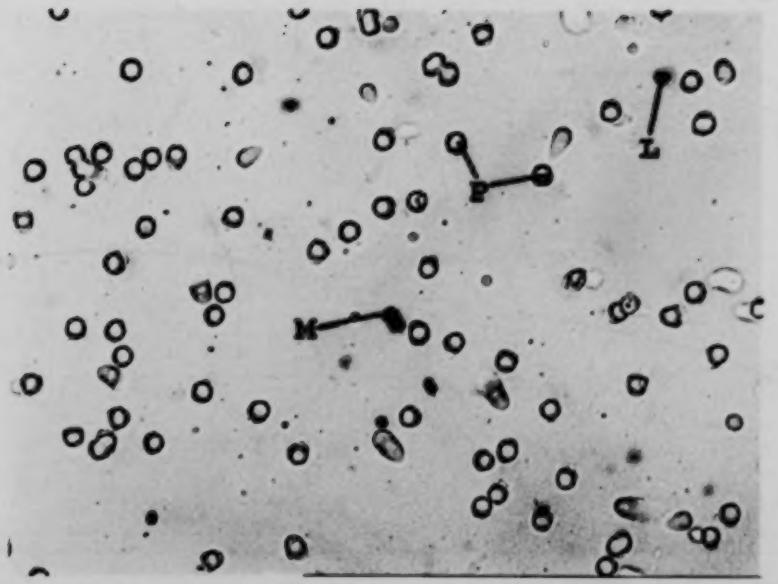

Figure 10C: Immunostain of Migrating PBMCs, From Normal Donors, on the Lower Surface of the Filter in the Migration Assay (Primary Antibody Deficient Negative Control). The filter was treated as outlined in Figures 11A and 11B except that no primary antibody was used. (L = lymphocyte, M= monocyte, P = pore)

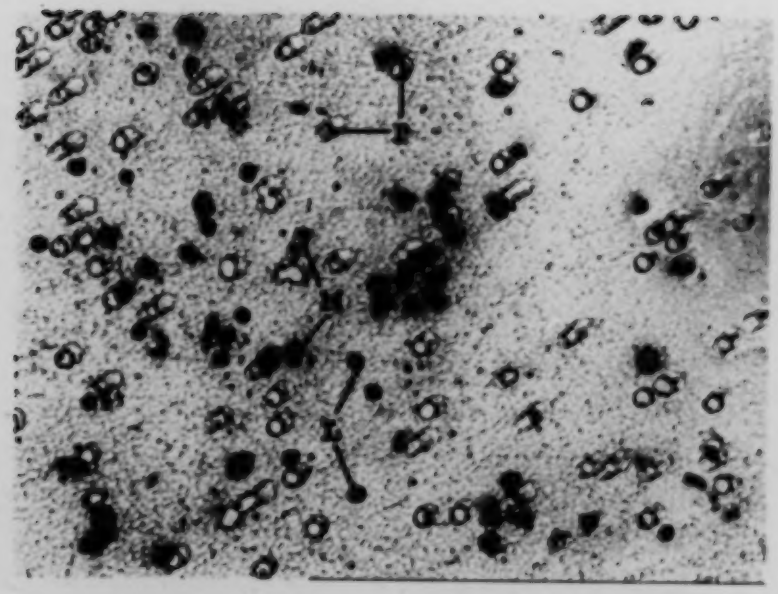

Figure 11A: Immunostain of Migrating PBMCs, From a Donor with Severe IHD, on the Lower Surface of the Filter in the Migration Assay. The filter was thin coated with ECM proteins for the migration assay. 300 000 PBMCs from a donor with severe IHD were placed in the upper well. The chamber was incubated for 2 hours in a 5% CO_2 incubator. Post-incubation, the upper surface of the filter was scraped and the adherent PBMCs on the lower surface of the polycarbonate filter were stained with an anti-CD14 antibody. Horseradish peroxidase conjugate and DAB substrate were used to develop positively stained cells. Cells were counterstained with hematoxylin. A positive reaction is indicated by brown staining with this method. (L = lymphocyte, P = pore, M= monocyte)

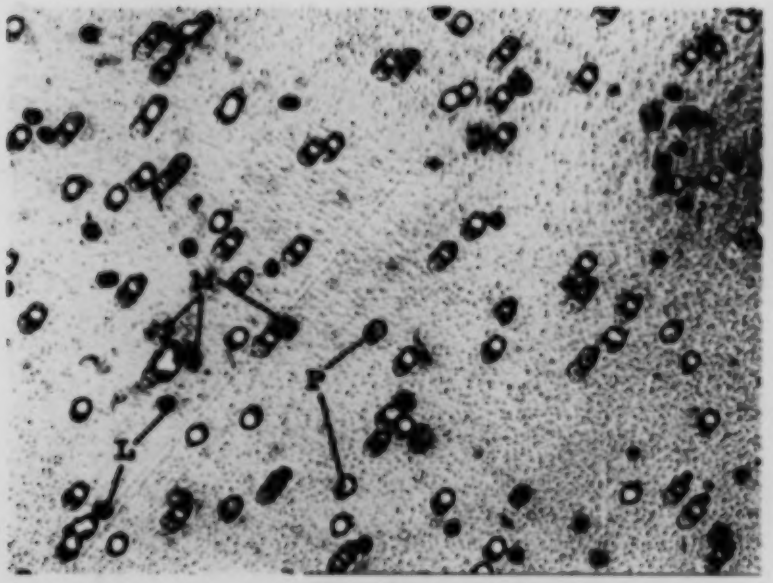

Figure 11B: Immunostain of Migrating PBMCs, From a Donor With Severe IHD, on the Lower Surface of the Filter in the Migration Assay (Anti-Isotypic Negative Control). The filter was thin coated with ECM proteins for the migration assay. 300 000 PBMCs from a donor with severe IHD were placed in the upper well. The chamber was incubated for 2 hours in a 5% CO_2 incubator. Post-incubation, the upper surface of the filter was scraped and the adherent PBMCs on the lower surface of the polycarbonate filter were stained with an anti-isotypic anti-rabbit anti-IgG antibody. Horseradish peroxidase conjugate and DAB substrate were used to develop positively stained cells. A positive reaction is indicated by brown staining with this method. Cells were counterstained with hematoxylin. (L = lymphocyte, M= monocyte, P = pore)

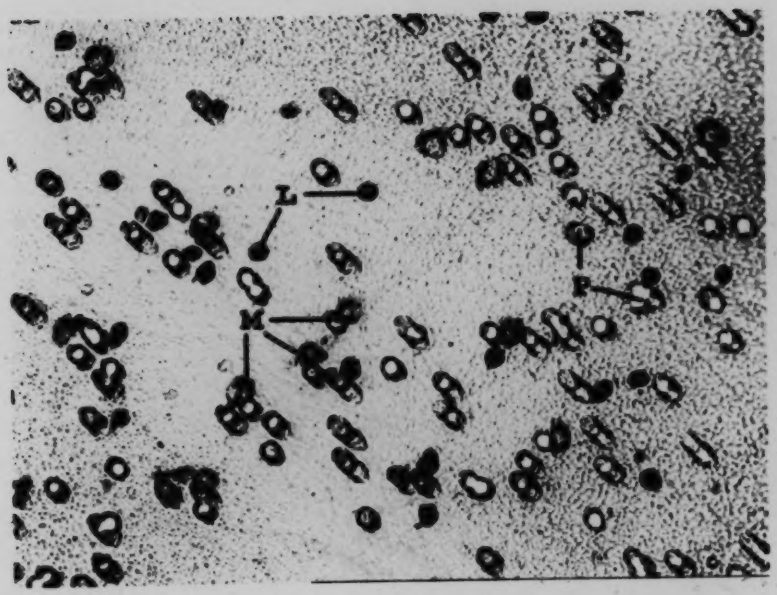

Figure 11C: Immunostain of Migrating PBMCs, From a Donor With Severe IHD, on the Lower Surface of the Filter in the Migration Assay (Primary Antibody Deficient Negative Control). This experiment was done as outlined in Figures 12A and 12B except that no primary antibody was used. (P = pore, M= monocyte, L = lymphocyte)

III.IIe. Monocyte Stimulation Experiments

The increase in monocytic migration in patients with severe IHD and rheumatoid arthritis suggests that these cells had increased levels of activation *in vivo*. To confirm whether this increase in migration was reflective of an increased level of activation of peripheral blood monocytes, lipopolysaccharide (LPS) stimulant was used in an attempt to increase the baseline monocyte migration rate from that of normal donors to the level of donors with heart disease. To achieve this, PBMCs were pre-treated with LPS for 30 minutes on ice, in each of 6 separate experiments, using blood from 5 normal donors and 2 donors with severe IHD. The pretreatment was done to allow for binding of LPS to its receptor prior to the migration assay. This was done on ice since activated monocytes readily bind to plastic, which leads to the loss of many monocytic cells. Maintenance of cells at low temperature prevents this from occurring.

When the migration rates from the individual stimulation experiments were averaged and compared to the baseline, a significant ($p=0.0222$ by ANOVA) increase in migration was found with the monocytoid cells from normal donors with LPS treatment (Figure 12a). Using the saline control as a baseline, differences were significant at 10 ng ($p=0.0084$ by Fisher PLSD) and at 100 ng doses of LPS ($p=0.0059$ by Fisher PLSD). Optimum stimulation for monocyte migration was at 100 ng LPS per well. While this stimulation was not to the level of the severe IHD patients, there was approximately a two fold increase at the optimum doses of LPS. No increase in migration occurred in monocytoid cells from patients with severe IHD (Figure 12b).

III.IIf. Potential Effects of Heart Medication on Migration Rates

To ensure that heart medications were not responsible for the increased monocyte migration rate in patients with severe IHD, every class of heart medication that heart patients were prescribed was tested for a correla-

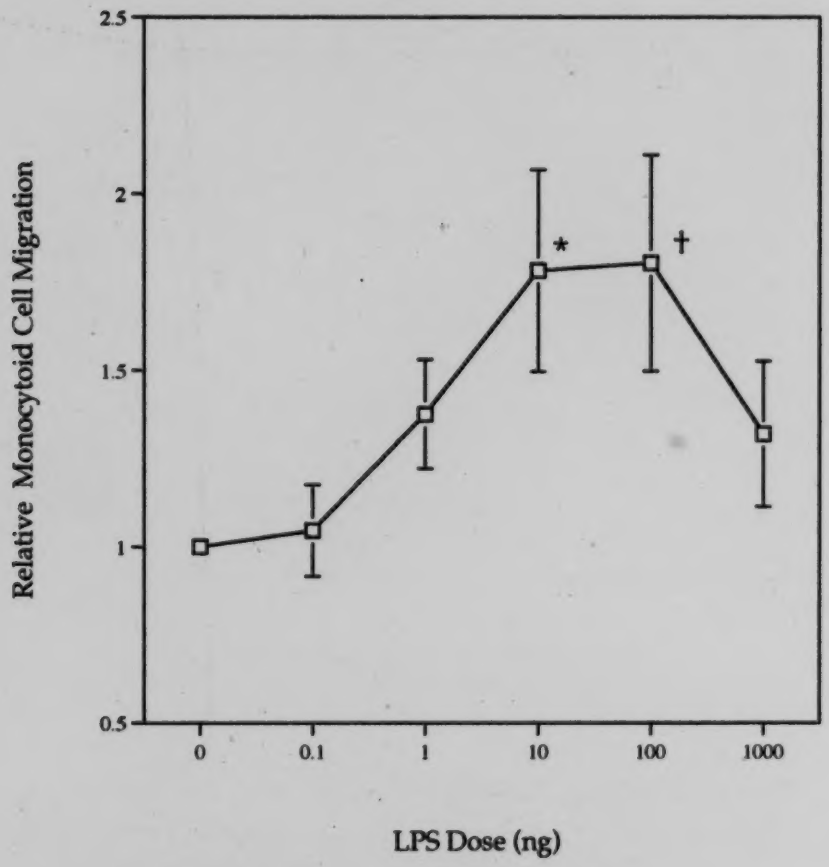

FIGURE 12A: Monocyte Stimulation Using Lipopolysaccharide (LPS) Stimulant (Normal Donor). For each experiment, 300 000 PBMCs were placed in the upper well. Post-incubation, monocytoid cells were enumerated based on cellular morphology with hematoxylin staining. To arrive at the cell count, 10 fields were counted per filter, with three filters counted per experimental condition. A total of five experiments from 5 separate donors were averaged. Monocytoid counts, from each dose of LPS, were divided by the cell counts in the control well to yield 'relative monocytoid cell migration'. As compared to the control well, *p=0.0084 by Fisher PLSD, †p=0.0059 by Fisher PLSD. Error bars were too small to be shown at 0 ng LPS.

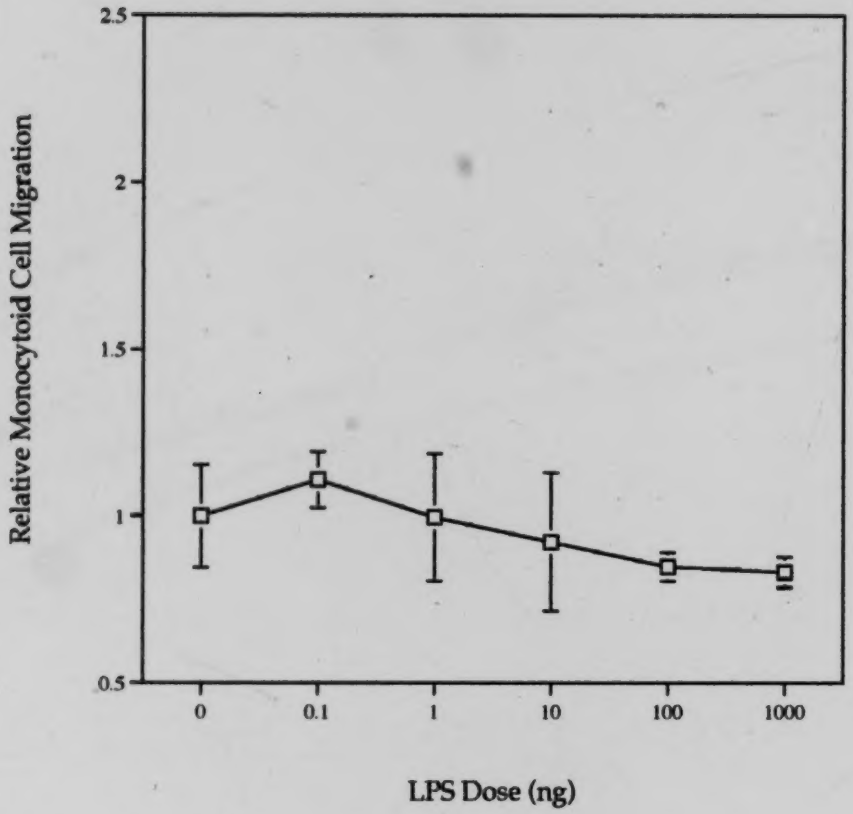

FIGURE 12B: Monocyte Stimulation Using Lipopolysaccharide (LPS) Stimulation (severe IHD) . Experiments were run, and normalization of data was done, as outlined in Figure 12A except that PBMCs were isolated from donors with severe IHD. Two experiments from two donors were averaged for each plotted point.

tion with the increased level of migration (see section III.If). No difference was found between the level of monocyte migration and the presence of any class of heart medications. These included: acetylsalicylic acid (p=.8179), nitroglycerin (p=.711), ß-blockers (p=.3127), ACE inhibitors (p=.7093), cholesterol lowering medications (p=.3541), calcium channel blockers (p=.8465), or parasympathetolytics (p=.4842). Because of this lack of correlation between any of the heart medications and the increased migration, and no differences being shown between monocyte migration rates from patients with moderate IHD and normal donors, it is clear that the differences in migration were not due to the effects of heart medications, or the treatment of the patients, nor the handling of the blood cells during the isolation procedure. The treatment of the patients may account for the small, non-significant increase in the lymphocytes however.

III.III. DISCUSSION

III.IIIa. Peripheral Blood Monocyte Isolation Procedure Development

The best method for the isolation of monocytes is counterflow centrifugation (Norris *et al* 1979, Sanderson *et al* 1977, Mason and Weiner 1985). Unfortunately, the equipment that is required for this is prohibitively expensive for most laboratories. Hence, most authors use a density centrifugation method where cells are separated based on their relative density. It was this type of method that was used as the protocol for partially purifying monocytes in this thesis.

When one uses a density centrifugation method, there is a requirement for taking precautions against the loss of monocytic cells by adherence to the holding vessels. Therefore, monocytes were kept in the cold during and after the isolation procedure. Otherwise, the loss of the monocytes by adherence of these cells to the sides of the tubes would be

risked. This should not be surprising. One monocyte purification method is to allow for the adherence of peripheral blood cells to plastic (Nielsen 1987, Kumagai *et al* 1979, Gadeberg *et al* 1979, Mantovani *et al* 1979). There are some major problems with the adherence method however. If cells are detached improperly, cell viability can be decreased (Gadeberg *et al* 1979, Mantovani *et al* 1979). This method can also potentially lead to the loss of a monocyte subpopulations (Nielsen 1990).

There has been some debate over the years at to whether it is better to use partially purified, or highly purified, cell populations due to the risk of losing a subpopulation of monocytes during further purification steps. Four studies have been done to address this in Boyden chamber based assays where cells were enumerated on the lower surface of the filter (Falk and Leonard 1980, Nielsen 1987, Zanella *et al* 1981, Leb *et al* 1983). In all of these studies an identical chemotactic function was demonstrated in the partially purified and adherence purified populations. Using the highly purified monocytes that can only be obtained by counterflow centrifugation, no difference was found between the highly purified monocytes and the partially purified monocytes which were obtained by density centrifugation (Diamond and Erickson 1982). This led Nielsen (1990) to suggest that it was not necessary to use highly purified monocytes in a Boyden chamber migration assay, although highly purified cells might be advantageous in measuring other functions.

In the collection of blood for the purpose of monocyte isolation, by density centrifugation, care should be taken in the selection of anticoagulant. A high level of heparin (80 IU/ml) has been shown to have an inhibitory effect on migration (Böyum 1968, Nielsen 1985), although the monocyte yield is excellent with both heparin and EDTA anticoagulants. Citrate, EGTA, and oxalate anticoagulants all have caused decreased monocyte yields (Nielsen 1985). Therefore, EDTA was chosen as the blood anticoagulant in these studies as it was the only anticoagulant that was readily available that did not affect migration, nor affect cell yields, adversely.

III.IIIb. Development of the Monocyte Migration Assay:

From earlier work with KG-1 and THP-1 cells, a new coating method was developed for the monocyte migration assay which relied on a thin coating of Matrigel over the upper surface of the filter. This coating likely contained many of the same protein constituents as a basement membrane. This is based on the composition of Matrigel, a complex, undefined mixture ECM proteins that are present in the basement membrane which include: collagen, laminin, fibronectin, and some growth factors (Kleinman *et al* 1982, McGuire and Seeds 1989). Cells that encountered these ECM constituents during the migration assay may experience what a cell normally encounters as it tries to penetrate through the basement membrane of an artery during transmigration, or when it contacts the exposed connective tissue that is often present in a damaged artery. This arterial damage and connective tissue exposure potentially arises with plaque denudation, or shortly after foam cell release from a fibrofatty lesion (Faggiotto *et al* 1984, Faggiotto and Ross 1984, Davies *et al* 1988, Davies 1996).

Because of the similarity between the protein component of plaque, and the composition of Matrigel, it is reasonable to exploit the use of a Matrigel coating to reflect *in vitro* what a cell could encounter *in vivo* in a damaged artery or area of advanced atherosclerosis. It is tempting to speculate that if a cell migrated over an ECM protein coating at an enhanced rate *in vitro*, it would reflect enhanced activity of that cell *in vivo*. Cells with higher migration rates in the migration assay, therefore, may have been 'primed' to play an active role in an ongoing inflammatory or immune response.

Since monocytes demonstrated increased migratory activity in the migration assay, it is tempting to speculate that the increase arose from a priming of the monocytes for a chronic inflammatory response which was caused by the presence of significant levels of unstable plaque in these

arteries. This hypothesis is supported by the high rate of monocyte migration both from patients with severe IHD and the patient with the chronic inflammatory disease rheumatoid arthritis (discussed in section III.IIIc).

At the very high dilutions of Matrigel that were used in the monocyte migration assay, a protein barrier was not produced over the pores. Instead, a thin ECM protein coating was created over the upper surface of the polycarbonate membrane which was too thin to obstruct the pores (section III.IIb). During the migration assay, the monocytes came in direct contact with this ECM coating which allowed the cells to penetrate through the filters in the absence of the addition of chemoattractants into the lower chamber. Otherwise, migration of monocytes onto the lower surface of the filter could not have occurred.

This approach contrasts with previously documented work where uncoated filters were used in Boyden chamber chemotaxis assays (Boyden 1962). With a thin coating of ECM proteins, a surface was presented to the cells which could potentially appear in areas of endothelial denudation or ruptured plaque *in vivo* where connective tissue may be directly exposed to the bloodstream (Davies *et al* 1988, Faggiotto *et al* 1984, Davies 1996, Fuster *et al* 1992). From the results of this study, we know that mononuclear cells are able to cross a membrane barrier which contains a thin ECM coating when no migration could occur in the absence of this coating. What resulted was the first documented case of significant monocyte migration in a two chamber assay in the absence of a chemoattractant which was added into the lower well.

The exact mechanism of the significant monocyte migration from heart patients is unknown. We do know though that monocyte migration has been tied to cellular activation by other authors (Josefsen *et al* 1994, Yano *et al* 1995, Locati *et al* 1994). The ECM protein coating on the upper surface of the filter provided a site for monocyte binding, possibly through the use of an ECM specific receptor, and it has previously been shown that the presence of the ECM protein collagen, types I and III, in both native and denatured forms,

can increase monocyte activity as exhibited by a marked increase in collagenase production (Shapiro *et al* 1992). Damaged ECM has also been shown to increase monocyte and T cell secretion of TNFα (Hershkoviz *et al* 1993). Further, we know that the protein coating is a requirement for monocyte migration since the lack of the coating results in no migration. What is less clear is the exact process that is involved. Substantial study will need to be done to elucidate the exact mechanism of the cellular migration.

III.IIIc. Use of the Monocyte Migration Assay to Test Different Heart Patients

Using the novel migration assay, the activity level of monocytes, and lymphocytes, from patients with ischemic heart disease was tested. No increase in lymphocyte migration was detected. However, as was discussed in section III.IIc, a substantial increase in the rate of monocyte migration occurred in patients with severe IHD which suggests that these monocytes were activated. This view is strengthened when one considers that increased migration also occurred with monocytes from a patient with the chronic inflammatory disease, rheumatoid arthritis and that other authors have found a link between monocyte migration and increased activation of circulating blood monocytes (Josefsen *et al* 1994). Therefore, the increased monocyte migration may be due to peripheral blood monocyte activation in the bloodstream of these patients.

Monocyte activation was likely initiated by an ongoing inflammatory response in the rheumatoid patient and the patients with severe IHD. As presented in section III.IIc, this is reflected by increased monocyte migration only in patients with substantial numbers of unstable plaques, as indicated by angiogram results and the stratification of clinical stability. Clinical instability on its own did not cause increased monocyte migration. Similarly, the presence of plaque without associated clinical instability was not enough for increased monocyte migration. A vasculopath though, who had extensive

peripheral plaque development, but not cardiac instability, also had high levels of monocyte migration. Taken together, these results strongly suggest that the increase in monocyte migration was due to the presence of significant numbers of unstable plaques. Should this be true, then the increased activity of the blood monocytes from patients with severe IHD was most likely caused by an ongoing inflammatory response in the large number of unstable plaques that were present in these heart patients. The importance of inflammation was reflected by similar migration between patients with severe IHD and the patient with rheumatoid arthritis. Together, these results lend support to the "Response to Injury" hypothesis of Ross (1986) and the growing belief that atherogenesis is a chronic inflammatory response.

III.IIId. Confirmation of Monocyte Attachment and Enumeration on the Matrigel Coated Filter

Monocytes have been identified for centuries based on distinctive morphological characteristics post-staining (by hematoxylin for example) including a horseshoe shaped nucleus and a greater than 1:1 cytoplasm to nucleus ratio. Over the last few decades however, new methods have been developed for leukocyte typing based on the staining of specific cellular markers. When one combines both morphological and cell marker staining methods, it is far less likely that one would wrongly identify a cell type. Because of this, monocytes in the migration assay were first stained by hematoxylin to identify the monocytic cells by their morphological characteristics and the results were confirmed by immunostaining.

A confirmation of the cell type becomes particularly important when one considers that B cells have been detected with atypical morphologies under certain disease conditions (Linda Pilarski, personal communication). Conversely, monocytes occasionally have a more round nuclear shape which can make the identification of cell types difficult (Nielsen 1990) if the

morphological staining is done in the absence of a secondary identification method such as immunostaining.

Even though the CD14 antigen cluster is the most commonly utilized target for monocyte immunostaining (Gown *et al* 1986), it is not a perfect marker. A subpopulation of granulocytes has exhibited CD14 expression (Weingarten *et al* 1993), and there have been reports of CD14 staining with B cells under certain conditions (Ozdemirli *et al* 1996, Newman *et al* 1993). The CD14 staining of granulocytes presents no problem in this assay since these cells were removed by the purification process and, if a few cells contaminated the PBMC mixture, the cells could easily be excluded from cell counts based on their distinctive morphology.

There is a theoretical possibility however, that B cells could be counted as monocytes in this assay since there have been B cells reported with atypical morphology under certain disease conditions and since B cells were part of the cell mixture that was placed in the upper well during the migration assay. There have also been reports of anti-CD14 antibodies binding to B cells (Ozdemirli *et al* 1996). However, at least in the case of chronic lymphocyte leukemia, lymphocytes with atypical morphology did not stain with the CD14 antibody (Newman *et al* 1993). Therefore, atypical morphologies and anti-CD14 staining do not necessarily coincide. Further, there have been no reports of B cells with atypical morphology in heart patients. Thus, when one considers the critical role of the monocyte in atherogenesis (see Chapter 1), and the lack of correlation between the presence of B cells in atherosclerotic plaque, most evidence points to increased monocyte migration in patients with severe IHD.

III.IIIe. Monocyte Stimulation Experiments

To confirm whether monocytes with higher migration rates have an associated increased level of activation, PBMCs were pretreated with LPS, a

monocyte stimulant. This was done to determine whether monocytes from normal donors, which should be non-activated in the bloodstream based on their low migration rates, could have their migration rates increased to the level of activated monocytes from donors with severe IHD. An increase in monocyte migration would demonstrate that the cells were not fully activated since the stimulant could raise the migration rates above baseline.

An increase in migration did occur with monocytes from the normal donors, although it was not to the level of monocytes from patients with severe IHD. When one considers however, the very short period of time that each cell had for that stimulation to occur, and the artificial nature of that stimulation *in vitro*, it is not entirely surprising that these same levels could not be achieved. When one considers that monocytes from the severe IHD donor could not be stimulated to migrate faster than the baseline, while the monocytes from the normal donor showed significant increases in migration, the monocytes from the severe IHD donor clearly behaved differently than their normal counterparts. This likely reflects that the cells from donors with severe IHD were fully activated in the bloodstream. This, in turn, would not allow any further activation of these cells by the LPS stimulant. When one combines the results from both the normal and severe IHD donors it appears that the increased migration in normal donors was because the cells were not previously activated. The results from these stimulation experiments, the comparison in cellular migration between different donor groups, and the results from previous authors which show that increased migration is tied to cellular activation (Jonesen *et al* 1994), together demonstrate that monocytes are more active in patients with severe IHD.

III.IIIf. Potential Effects of Heart Medications on Migration Rates

When an increased level of migration was found between patients with IHD and normal donors, a prime concern was to elucidate whether this

was due to heart disease or some other factor such as heart medication. As is discussed in section III.IIf, when the migration rates were compared against the prescribed heart medications, no correlation was found between the presence of any class of heart medication and the increased monocyte migration. When one considers that monocytes from the heart patients with moderate IHD shared identical treatment to the monocytes from patients with severe IHD, it appears likely that the differences in monocyte migration arose solely from differences in the numbers and stability of the atherosclerotic plaques that were present in these patients.

CHAPTER IV - SUMMARY

IV.I. DISCUSSION OF OBJECTIVES

As stated in section I.VIa., the objectives of this thesis were as follows:

1) To develop an assay for testing the level of activation, specifically the migratory capacity of primary isolates of circulating peripheral blood monocytes, which mimics the environment of plaque.

2) To use this assay to determine whether monocytes from patients with unstable coronary arterial atherosclerotic plaque development have increased levels of cellular activity (as reflected by the rate of migration).

With regards to the first objective, the migration assay that was developed mimicked certain aspects of what a monocyte would encounter in a region of unstable plaque development, where connective tissue is often exposed to the bloodstream (Faggiotto *et al* 1984, Faggiotto and Ross 1984, Davies 1988). This was achieved by exposing the PBMCs to Matrigel, a complex, undefined mixture of proteins that comprise a basement membrane. One should recognize however that the arterial environment is not reproduced perfectly. There was no blood flow over the connective tissue proteins. Further, the complex mixture of activated cells, growth factors, and inflammatory factors, from the environment of plaque, was not reproduced. Therefore, the first objective of this thesis was only partially achieved.

The second objective of the thesis was clearly met. PBMCs were tested for increased activity in patients with heart disease. A significant difference in monocyte activity was detected in monocytes from patients with severe

IHD, as compared to controls, while no differences were found in lymphocytes from the same blood donors.

IV.II. CONCLUSIONS FROM THIS STUDY

The primary conclusions that were derived from this study follow:

1) A modified two-chamber migration assay was developed where the rate of migration appeared to reflect the overall level of monocyte activation.

2) Differences in the pattern of cellular activity were found in studies which used PBMCs from heart patients with unstable coronary syndromes and normal donors.

3) LPS stimulated an increase in monocytoid cell migration using PBMCs from normal donors, but did not increase migration in monocytoid cells using patients with severe IHD.

4) In the comparative studies between the two categories of heart patients and normal controls, the increase in the rate of monocyte migration, using PBMCs from patients with severe IHD, was not caused by the effect of any heart medication.

5) The increase in monocyte migration most likely arose from the unstable, inflammatory nature of the plaques in these patients.

IV.III. OUTLOOK FOR FUTURE STUDIES

There are several directions that I would like to see this work taken in the future:

1) The results of this study should be confirmed in a second assay system. One approach, which has a high chance of success, is to test for differences in monocyte migration using endothelial cells over a thick

Matrigel coating. This idea, which arose in committee meetings, merits considerable study. The study could be approached using glass slides, where migration would be viewed by confocal microscopy, or by using two chamber migration assays where endothelial cells would be grown on a Matrigel coating over a filter, and the cells could be allowed to migrate through the bottom of the filter. In a two chamber assay, the differences in cell migration could be compared by counting the number of adherent cells on the bottom of the filter or, if radiolabeling was selected as the enumeration method, on all cells that transited from the upper chamber through the endothelial cell/ECM protein/filter barrier and into the lower well.

Differences have been detected between different classes of endothelial cells including between coronary artery endothelial cells and human umbilical vein endothelial cells. Therefore, in the selection of the type of the type of cells for use as a monolayer between the two chambers, endothelial cells from coronary arteries would be preferred in these studies since they are the type of endothelial cell that would be present in areas of atherogenesis.

2) Phagocytosis has been used in the past to test for the level of phagocyte activation. Isolation of monocytic cells, followed by the phagocytosis of FITC labeled *Escherichia coli* or *Staphlococcus aureus* bacteria has been tied to cellular activity in the past. Bassøe *et al* (1983) have outlined one such method. Testing for the level of phagocytosis using freshly isolated monocytes from heart patients, and comparing levels of phagocytosis to normal controls, could provide a technically non-demanding way of comparing the level of activity of the blood cells.

3) The detection of increased levels of soluble CD14 in human serum has been tied to monocyte activation in Psoriasis (Schopf *et al* 1993) and in Behçet's Disease (Sahin *et al* 1996) using ELISA (enzyme

linked immunosorbent assay) assays. Testing for an increase in the level of soluble CD14 in the serum of patients with severe IHD would provide strong supportive evidence for the increased activation of peripheral blood monocytes in these patients.

4) It would be useful to elucidate whether the migration assay could be used to test if monocyte migration could be inhibited or stimulated by various substances that act directly on the cells. This could be useful for testing the effects of new pharmaceuticals on monocytes, or other leukocytes, in atherogenesis or in other inflammatory conditions.

5) Finally, the migration assay could prove to be a new effective method for testing the innate activity of monocytes under several disease and activation conditions. It could also prove to be effective as a means to test the activity of other important inflammatory cell types such as the polymorphonuclear leukocyte.

CHAPTER V - LITERATURE CITED

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